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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (e).

Express Mail Label No.

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☐ Additional inventors are being named on the _____ separately numbered sheets attached hereto**TITLE OF THE INVENTION (280 characters max)**

ACE2 ACTIVATION FOR TREATMENT OF HEART AND KIDNEY DISEASE AND HYPERTENSION

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ENCLOSED APPLICATION PARTS (check all that apply)☒ Specification Number of Pages

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☐ Other (specify)☒ Application Data Sheet. See 37 CFR 1.76**METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT**☐ Applicant claims small entity status. See 37 CFR 1.27.☐ A check or money order is enclosed to cover the filing feesFILING FEE
AMOUNT (\$)☒ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number:

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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Respectfully submitted,

SIGNATURE

Date

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Docket Number:

10723-43

TYPED or PRINTED NAME

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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Initial Information Data Sheet

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BERESKIN & PARR

U.S. PROVISIONAL PATENT APPLICATION

206T90-60468E09

**Title: ACE2 Activation for Treatment of
Heart and Kidney Disease and
Hypertension**

**Inventor(s): Michael A. Crackower and Josef M.
Penninger**

ACE2 Activation for Treatment of Heart and Kidney Disease and Hypertension

FIELD OF THE INVENTION

The present invention provides compositions and methods for use in diagnosing
5 and treating heart and kidney diseases, including hypertension, coronary heart disease, and heart and kidney failure.

BACKGROUND OF THE INVENTION

Cardiovascular disease will be the number one health care burden of the 21st
century, and is predicted to be the most common cause of death worldwide by
10 2020¹. A major risk factor for heart disease is high blood pressure². Hypertension is a multifactorial quantitative trait controlled by both genetic and environmental factors³. While much is known about environmental factors that can contribute to high blood pressure, such as diet and physical activity, less is known about the genetic factors that are responsible for predisposition to cardiovascular disease.
15 Despite the identification of several putative genetic quantitative trait loci (QTL) associated with hypertension in animal models⁴, none of these loci have been translated into genes⁵. Thus, the molecular and genetic mechanisms underlying hypertension and other cardiovascular diseases remain largely obscure.

One critical regulator of blood pressure homeostasis is the renin-angiotensin
20 system (RAS). The protease renin cleaves angiotensinogen into the inactive decaemic peptide angiotensin I (AngI). The action of angiotensin-converting enzyme (ACE) then catalyzes the cleavage of the AngI into the active octomer angiotensin II (AngII), which can contribute to hypertension by promoting vascular smooth muscle vasoconstriction and renal tubule sodium
25 reabsorption^{6,7}. ACE mutant mice display spontaneous hypotension, partial male infertility, and kidney malformations^{8,9}. In humans, an ACE polymorphism has been associated with determinants of renal and cardiovascular function¹⁰, and

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pharmacological inhibition of ACE and AngII receptors are effective in lowering blood pressure¹¹ and kidney disease¹². In addition, inhibition of ACE and AngII receptors has beneficial effects in heart failure¹³.

5 Recently a homologue of ACE, termed ACE2, has been identified which is predominantly expressed in the vascular endothelial cells of the kidney and heart^{14,15}. Interestingly, two ACE homologues also exist in flies¹⁶. Unlike ACE, ACE2 functions as a carboxypeptidase, cleaving a single residue from AngI, generating Ang1-9^{14,15}, and a single residue from AngII to generate Ang1-7¹⁴. These *in vitro* biochemical data suggested that, ACE2 modulates the RAS and
10 thus may play a role in blood pressure regulation. The *in vivo* role of ACE2 in the cardiovascular system and the RAS is not known.

Acton et al. in U.S. Patent 6,194,556, describe the use of ACE2 in diagnosis and therapeutics of ACE2 associated states. It is stated that ACE2 expression levels increase with hypertension and that antagonists or inhibitors of ACE2 activity
15 would be useful in the treatment of increased blood pressure or related disorders. Canadian patent application no. 2,372,387 provides specific examples of ACE2 inhibitors which are intended to be useful for treatment of heart disease such as hypertension. This again emphasizes the need to inhibit, rather than increase, ACE2 activity. The references teaching the need to inhibit ACE2 activity are
20 largely based only on *in vitro* experimental data. They do not provide *in vitro* data, such as knock out mammal data, to characterize ACE2. To date, no ACE2 inhibitors have been approved as pharmaceuticals for treatment of hypertension. Furthermore, the *in vivo* role of ACE2 in the cardiovascular system and the RAS remains largely unknown. There remains a need to characterize the function of
25 ACE2 in order to be able to design appropriate diagnostic tests and pharmaceuticals for treatment of heart and kidney disease.

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SUMMARY OF THE INVENTION

The invention provides a new paradigm for the regulation of the renin-angiotensin system and shows a completely new and unexpected usage of ACE2: it is a critical negative regulator of the RAS required for heart function and blood pressure control. Activation of ACE2 is critical for treatment and prevention of heart and kidney disease. The invention shows for the first time that administering an ACE2 activator to a subject prevents and treats hypertension and cardiac and kidney disease.

10 BRIEF DESCRIPTION OF THE DRAWINGS

Preferred embodiments of the invention will be described in relation to the drawings in which:

Figure 1. Sequence and chromosomal mapping of rat ACE2.

- a, Protein alignment of rat, mouse and human ACE2, with mouse and human testis-ACE (T-ACE). Blackened shading indicates amino acid identity and gray shading indicates degree of amino acid similarity. b, Schematic domain structure of ACE and ACE2. Note that ACE2 only contains one ACE-domain with the consensus zinc binding site HEMGH. The catalytic centers are indicated in blue, signal peptide is indicated in orange and transmembrane domain in purple. c, Expression patterns of mouse and rat ACE2 genes in different adult tissues and different days of embryonic development (E7 = embryonic day 7). Note that two isoforms are present for ACE2 in mice, but not in rat or human (not shown), a feature similar to that seen for ACE¹⁵. d, Results of radiation hybrid mapping of rat ACE2, compared to the mapping of a QTL identified in Sabra salt-sensitive animals (SS-X), SHRSP (BP3), and SHR rats (BB.Xs). Polymorphic marker

names are indicated to the left of the Ideogram. LOD scores and theta values for markers linked to *ACE2* are shown. cR = centiRads.

Figure 2. Expression levels of *ACE2* in rat models of hypertension.

- a*, Northern blot analysis of *ACE2* mRNA from kidneys of Sabra SBH/y and SBN/y rats. Upper section shows representation of Northern blots with actin control levels. Lower panel shows relative levels of *ace2* message normalized to actin levels. *b*, Western blot analysis of *ACE2* protein levels from kidneys of Sabra SBH/y and their control SBN/y rats, as well as SHR and SHRSP and their control WKY rats. Upper section shows representative Western blots. Systolic blood pressure (BP) in mmHg for the respective Sabra rats is indicated. Lower panel shows relative protein levels of *ACE2* corrected for actin. Bars show mean values \pm SEM. * = $p < 0.05$, ** = $p < 0.01$. (n=4, for all groups).

Figure 3. Targeted disruption of mouse *ACE2* by homologous recombination.

- a*, Gene targeting strategy. A portion of the murine *ace2* wild-type locus (top) is shown. Black boxes indicate exons. The targeting vector was designed to replace exon 9 encoding the zinc binding catalytic domain with the neomycin (neo) resistance gene cassette placed in the anti-sense orientation. Thymidine kinase (TK) was used for negative selection. The 3' and 5' flanking probes used for Southern analysis are indicated in dark blue. *b*, Southern blot analysis of *ace2^{+/y}* and *ace2^{-y}* ES cells. Genomic DNA was digested with *EcoRI* and hybridized to the 3' and 5' flanking probe shown in (a). *c*, Western blot analysis of *ACE2* protein expression in the kidneys of *ace2^{+/y} ace2^{-y}* mice. The anti-*ACE2* Ab is reactive to a region N-terminal to the deletion. *d*, RT-PCR analysis of *ACE* mRNA expression in the heart and kidneys of *ace2^{+/y}* and *ace2^{-y}* mice. Different PCR cycles for linear amplification and GAPDH mRNA levels as a control are shown.

Figure 4. Normal blood pressure and kidney functions

- a*, Blood pressure measurements in 3 month old *ace2^{+/y}* (n= 8) and *ace2^{-y}* (n = 8) mice in the absence (left panels) or presence of the ACE blocker captopril. Blood pressures were determined by using tail cuffing and mean values +/- SD are shown. captopril was administered to mice for 2 weeks prior to blood pressure measurements as described in Methods. These blood pressures were confirmed using invasive hemodynamic measurements (not shown). The differences in both captopril-treated *ace2^{+/y}* and *ace2^{-y}* mice are significantly different to that of their respective untreated groups ($\sim p < 0.01$). *b*, Normal kidney histologies in 6 month old *ace2^{+/y}* and *ace2^{-y}* mice. Arrows indicate glomeruli. *c,d*, Serum parameters for kidney functions in 6 month old *ace2^{+/y}* and *ace2^{-y}* mice. We failed to observe any significant changes in these parameters at all age groups analyzed. Moreover, there was no difference in the male or female groups (not shown). Mean values +/- SD are shown. (n=6, for both groups).

Figure 5. Heart morphology

- a*, H&E stained sections of hearts isolated from 6 month old *ace2^{+/y}* and *ace2^{-y}* mice. Note the enlarged left ventricles (LV) and right ventricles (RV) in *ace2^{-y}* mice. However, the overall heart size is comparable between both genotypes and there is no evidence for cardiac hypertrophy macroscopically or in isolated cardiomyocytes. *b*, Quantitation of heart/body weight ratios from 6 month old *ace2^{+/y}* (n= 8) and *ace2^{-y}* (n=8) mice as an indicator of cardiac hypertrophy. It should be noted that the body weights, heart weights, tibial lengths, and the heart weight/tibial length ratios were also not changed between the different genetic groups at all ages analyzed (not shown). *c,d*, Absence of interstitial fibrosis in *ace2^{-y}* mice. One hallmark feature for dilated cardiomyopathy is interstitial fibrosis. However, interstitial fibrosis was comparable between hearts of *ace2^{+/y}* (n= 8) and *ace2^{-y}* (n=8). (c) shows PSR staining of individual hearts. Note the normal perivascular fibrosis, stained in red, in both wild type and mutant animals. (d) quantitation of fibrotic changes in the interstitium.

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Figure 6. Loss of ACE2 results in severe contractile heart failure

- a, Echocardiographic measurements of contracting hearts in a 6 months old *ace2^{+/y}* and two *ace2^{-y}* mice. Peaks and valleys indicate the systole and diastole of individual heart beats. Arrows indicate the distance between systolic contraction (LVESD) and diastolic relaxation (LVEDD), values that determine the percentage of fractional shortening (% FS). Note the increased diastolic and systolic dimensions in the *ace2^{-y}* mice indicative of cardiac dilation. b, Percentage fractional shortening and velocity of circumferential fibre shortening, two hallmark parameters for heart contraction, in 6 month old *ace2^{+/y}* (n=8) and *ace2^{-y}* (n=8) mice and 6 month old *ace2^{+/-}* (n=5) and *ace2^{-/-}* (n=5) female mice. Values were determined by echocardiography. Mean values +/- SD are shown. * p < 0.05 and ** p < 0.01 between genetic groups. c, Blood pressure measurements in 6 month old male *ace2^{+/y}* (n = 8) and *ace2^{-y}* (n = 8) and 6 months old female mice *ace2^{+/-}* (n = 5) and *ace2^{-/-}* (n = 5) female mice. Mean values +/- SD are shown. Blood pressures were confirmed using invasive hemodynamic measurements. * p < 0.05.

Figure 7. Upregulation of hypoxia markers and increased angiotensin II levels in the absence of ACE2

- a,b, Northern blot analysis of BNIP3 and PAI-1 mRNA expression levels, two hypoxia-inducible genes in 6 month old male *ace2^{+/y}* and *ace2^{-y}* mice. (a) shows individual Northern blot data and (b) relative levels of BNIP3 and PAI-1 mRNA levels normalized to the gapdh control. ** p<0.01. (n=5)
- c, AngiotensinI (AngI) and AngiotensinII (AngII) peptide levels in the heart and kidneys of 6 month old male *ace2^{+/y}* (n = 8) and *ace2^{-y}* (n = 8) littermate mice. AngI and AngII tissue levels were determined by radioimmunoassays. Mean peptide levels +/- SD are shown. ** p<0.01.

Figure 8. ACE-ACE2 double mutant mice do not develop heart failure

5 a, Blood pressure measurements in 6 month old male *ace2^{+/y}* (n = 8), *ace^{-/-}* (n = 8), and *ace^{-/-} ace2^{+/y}* double mutant (n = 6) mice. Mean values +/- SD are shown. Blood pressures were confirmed using invasive hemodynamic measurements. ** p<0.01 of mutant as compared to *wild type* mice. b, Percentage fractional shortening and velocity of circumferential fibre shortening in 6 month old male *ace2^{+/y}* (n = 8), *ace2^{-/-}* (n = 8), *ace^{-/-}* (n = 8) and *ace^{-/-} ace2^{+/y}* double mutant (n = 6) littermates. Values were determined by echocardiography. Mean values +/- SD are shown. ** p < 0.01 between genetic groups. c, Echocardiographic measurements of contracting hearts in a 6 month old male *ace2^{+/y}*, *ace2^{-/-}*, *ace^{-/-}*, and *ace^{-/-} ace2^{+/y}* double mutant littermate mice. Echocardiograms were analyzed and are labeled as in Figure 6a. Note the ablation of ACE in an *ace2* null background completely rescues the contractile heart defects observed in *ace2^{+/y}* single mutant mice.

15 Figure 9. a, Human ACE2 DNA (SEQ ID NO:1). b, Human ACE2 polypeptide (SEQ ID NO:2).

Figure 10. a, Mouse ACE2 DNA (SEQ ID NO:3). b, Mouse ACE2 polypeptide (SEQ ID NO:4)

Figure 11. ACE2 nucleotide polymorphisms and sequences.

DETAILED DESCRIPTION OF THE INVENTION

20 The invention provides a new paradigm for the regulation of the renin-angiotensin system and shows that ACE2 is a critical negative regulator of the RAS required for heart function and cardiovascular function. Activation of ACE2 is critical for treatment and prevention of heart and kidney disease and hypertension. The invention shows for the first time that administering an ACE2 activator to a
25 subject prevents and treats heart failure and hypertension.

This result is completely unexpected in view of prior art references, such as US Patent No. 6,194,556 and Canadian patent application no. 2,372,387, described above, that teach that ACE2 activity must be *inhibited* in order to treat heart disease. It is thus surprising that heart disease is actually treated by *activating*

5 ACE2 expression and/or activity.

The invention includes activators that induce but are not limited to activators of ACE2 function and/or ACE2 mRNA and ACE2 protein expression and pharmaceutical compositions including the activators. The invention also includes methods of medical treatment of heart disease and kidney disease and

10 hypertension by administration of an effective amount of an activator to a subject in need of treatment.

The invention also includes screening assays for detecting ACE2 activators which may be used to treat heart disease and kidney disease and hypertension. These assays are *in vitro* or *in vivo*. In a preferred embodiment, the invention

15 includes an endothelial, kidney or heart cell assay for evaluating whether a candidate compound is capable of increasing ACE2 expression or activity. Cells are cultured in the presence of at least one compound whose ability to activate expression or activity is sought to be determined and the cells are the cells for an increase in the level of ACE2 expression. Another aspect of the invention

20 involves an ACE2 knock-out mouse for identifying compounds that may overcome the effects of loss of ACE2. Polypeptides and small organic molecules are tested in these assays. The invention includes all compounds that are identified with the screening methods of the invention and which are suitable for administration to subjects in pharmaceutical compositions.

25 Another aspect of the invention is diagnosis of the onset or risk of heart and/or kidney disease and/or hypertension. This may be diagnosed by measuring ACE2 levels in heart, serum, or kidney, or other tissues. Levels of ACE2 less than wild type levels are indicative of an "ACE2 decreased state" which this invention

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shows is directly connected with heart and/or kidney disease and/or hypertension, or a risk of disease. An ACE2 decreased state is also indicated by the polymorphisms ACE2a-ACE2m described below. Diagnosis is also optionally accomplished by analysis of polymorphisms upstream and downstream of and within the ACE2 gene which are associated with an ACE2 reduced state. All the reagents required for the detection of nucleotide(s) that distinguish the polymorphisms, by means described herein, can be provided in a single kit for analysis of isolated genomic DNA from a subject. The kit would contain labelled probes that distinguish polymorphisms of ACE2 in order to allow genotyping and phenotyping, for diagnosis of risk or onset of disease. Polymorphism-specific probes can be appropriately labelled and added to the generated DNA segments under annealing conditions, such that only one of the polymorphism-specific probes hybridizes and can be detected, thereby identifying the specific ACE2 polymorphism.

15 **Therapeutic Methods**

As hereinbefore mentioned, the present inventors have shown that ACE2 gene expression is down-regulated in hypertension, heart and kidney disease. Accordingly, the present invention provides a method of treating or preventing hypertension, heart disease or kidney disease comprising administering an effective amount of an agent that can increase the expression of ACE2 to an animal in need thereof.

The term "an agent that can increase the expression of ACE2" as used herein means any agent that can increase the level or activity of an ACE2 gene or protein as compared to the level or activity of the ACE2 gene or protein in the same type of cell in the absence of the agent. The agent can be any type of substance including, but not limited to, nucleic acid molecules, proteins, peptides, carbohydrates, small molecules, or organic compounds. Whether or not the ACE2 gene is increased can be readily determined by one of skill in the art using

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known methods including Western blotting SDS-PAGE, immunochemistry, RT-PCR, Northern blotting and in situ hybridization.

The term "animal" as used herein includes all members of the animal kingdom. The animals are preferably human.

- 5 The term "effective amount" as used herein means an amount effective at dosages and for periods of time necessary to enhance the level of ACE2.

- The term "treatment or treating" as used herein means an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of
- 10 one or more symptoms or conditions, diminishment of extent of disease, stabilized (i.e. not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, and remission (whether partial or total), whether detectable or undetectable. "Treating" can also mean prolonging survival as compared to
- 15 expected survival if not receiving treatment.

Administration of ACE2 nucleic acid molecule

In one embodiment, the expression of the ACE2 gene is increased by administering a nucleic acid that comprises an ACE2 gene or portion thereof.

- In another embodiment, the expression of the ACE2 gene may be increased by
- 20 administering an agent that increases ACE2 gene expression including any agents identified using the screening assays in this application.

Since a subject suffering from disease, disorder or abnormal physical state can be treated by up regulation of ACE2, gene therapy to increase ACE2 expression is useful to modify the development/progression of heart or kidney disease.

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The invention includes methods and compositions for providing ACE2 gene therapy for treatment of diseases, disorders or abnormal physical states characterized by decreased ACE2 expression or levels of activity of ACE2 polypeptide.

- 5 The invention includes methods and compositions for providing a nucleic acid molecule encoding ACE2 or functionally equivalent nucleic acid molecule to the cells of a subject such that expression of ACE2 in the cells provides the biological activity or phenotype of ACE2 polypeptide to those cells. Sufficient amounts of the nucleic acid molecule are administered and expressed at
- 10 sufficient levels to provide the biological activity or phenotype of ACE2 polypeptide to the cells. For example, the method can preferably involve a method of delivering a nucleic acid molecule encoding ACE2 to the cells of a subject having heart or kidney disease, comprising administering to the subject a vector comprising DNA encoding ACE2. The method may also relate to a
- 15 method for providing a subject having heart or kidney disease with biologically active ACE2 polypeptide by administering DNA encoding ACE2. The method may be performed *in vivo* or *ex vivo* (eg. with heart or kidney stem, progenitor cells or other cells to be transplanted cells). Methods and compositions for administering ACE2 (including in gene therapy) to isolated cell or a subject are
- 20 explained, for example, in U.S. Patent Nos. 5,672,344, 5,645,829, 5,741,486, 5,656,465, 5,547,932, 5,529,774, 5,436,146, 5,399,346, 5,670,488, 5,240,84, 6,322,536, 6,306,830 and 6,071,890 and US Patent Application Nos. 20010029040 which are incorporated by reference in their entirety.

- The method also relates to a method for producing a stock of recombinant virus
- 25 by producing virus suitable for gene therapy comprising DNA encoding ACE2. This method preferably involves transfecting cells permissive for virus replication (the virus containing the nucleic acid molecule) and collecting the virus produced.

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The methods and compositions can be used *in vivo* or *in vitro*. The invention also includes compositions (preferably pharmaceutical compositions for gene therapy). The compositions include a vector containing ACE2. The carrier may be a pharmaceutical carrier or a host cell transformant including the vector.

- 5 Vectors known in the art include but are not restricted to retroviruses, adenoviruses, adeno associated virus (AAV), herpes virus vectors, such as vaccinia virus vectors, HIV and lentivirus-based vectors, and plasmids. The invention also includes packaging and helper cell lines that are required to produce the vector. Methods of producing the vector and methods of gene
10 therapy using the vector are also included with the invention.

The invention also includes a transformed cell containing the vector and the recombinant ACE2 nucleic acid molecule sequences.

Use of ACE2 Variants - Modifications to polypeptide sequence

- ACE2 variants may be used in methods of the invention. Changes which result
15 in production of a chemically equivalent or chemically similar amino acid sequence are included within the scope of the invention. Polypeptides having sequence identity to ACE2 receptor are tested to ensure that they are suitable for use in the methods of the invention. Variants of the polypeptides of the invention may occur naturally, for example, by mutation, or may be made, for example,
20 with polypeptide engineering techniques such as site directed mutagenesis, which are well known in the art for substitution of amino acids. For example, a hydrophobic residue, such as glycine can be substituted for another hydrophobic residue such as alanine. An alanine residue may be substituted with a more hydrophobic residue such as leucine, valine or isoleucine. A negatively charged
25 amino acid such as aspartic acid may be substituted for glutamic acid. A positively charged amino acid such as lysine may be substituted for another positively charged amino acid such as arginine.

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Therefore, the invention includes polypeptides having conservative changes or substitutions in amino acid sequences. Conservative substitutions insert one or more amino acids, which have similar chemical properties as the replaced amino acids. The invention includes sequences where conservative substitutions are
5 made that do not destroy compound activity.

Polypeptides comprising one or more d-amino acids are contemplated within the invention. Also contemplated are polypeptides where one or more amino acids are acetylated at the N-terminus. Those with skill in the art recognize that a variety of techniques are available for constructing polypeptide mimetics with the
10 same or similar desired compound activity as the corresponding polypeptide compound of the invention but with more favorable activity than the polypeptide with respect to solubility, stability, and/or susceptibility to hydrolysis and proteolysis. See, for example, Morgan and Gainor, Ann. Rep. Med. Chem., 24:243-252 (1989). Examples of polypeptide mimetics are described in U.S.
15 Patent Nos. 5,643,873. Other patents describing how to make and use mimetics include, for example in, 5,786,322, 5,767,075, 5,763,571, 5,753,226, 5,683,983, 5,677,280, 5,672,584, 5,668,110, 5,654,276, 5,643,873. Mimetics of the polypeptides of the invention may also be made according to other techniques known in the art. For example, by treating a polypeptide of the invention with an
20 agent that chemically alters a side group by converting a hydrogen group to another group such as a hydroxy or amino group. Mimetics preferably include sequences that are either entirely made of amino acids or sequences that are hybrids including amino acids and modified amino acids or other organic molecules.

25 The invention also includes hybrid and polypeptides, for example where a nucleotide sequence is combined with a second sequence.

The invention also includes methods of using polypeptide fragments of ACE2 which may be used to confer compound activity if the fragments retain activity.

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The invention also includes polypeptides fragments of the polypeptides of the invention which may be used as a research tool to characterize the polypeptide or its activity. Such polypeptides preferably consist of at least 5 amino acids. In preferred embodiments, they may consist of 6 to 10, 11 to 15, 16 to 25, 26 to 50, 51 to 75, 76 to 100 or 101 to 250 or 250 to 500 amino acids. Fragments may include sequences with one or more amino acids removed, for example, C-terminus amino acids in a compound sequence.

Enhancement of ACE2 polypeptide activity

The activity of ACE2 is increased or decreased by carrying out selective site-directed mutagenesis. A DNA plasmid or expression vector containing the nucleic acid molecule or a nucleic acid molecule having sequence identity is preferably used for these studies using the U.S.E. (Unique site elimination) mutagenesis kit from Pharmacia Biotech or other mutagenesis kits that are commercially available, or using PCR. Once the mutation is created and confirmed by DNA sequence analysis, the mutant polypeptide is expressed using an expression system and its activity is monitored.

The invention also includes methods of use of polypeptides which have sequence identity at least about: >20%, >25%, >28%, >30%, >35%, >40%, >50%, >60%, >70%, >80% or >90% more preferably at least about >95%, >99% or >99.5%, to human or mouse ACE2 (or a partial sequence thereof). Modified polypeptide molecules are discussed below. Preferably about: 1, 2, 3, 4, 5, 6 to 10, 10 to 25, 26 to 50 or 51 to 100, or 101 to 250 nucleotides or amino acids are modified.

Identity is calculated according to methods known in the art. Sequence identity is most preferably assessed by the BLAST version 2.1 program advanced search (parameters as above). BLAST is a series of programs that are available online at <http://www.ncbi.nlm.nih.gov/BLAST>. The advanced blast search

(<http://www.ncbi.nlm.nih.gov/blast/blast.cgi?Jform=1>) is set to default parameters. (ie Matrix BLOSUM62; Gap existence cost 11; Per residue gap cost 1; Lambda ratio 0.85 default).

- References to BLAST searches are: Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403_410; Gish, W. & States, D.J. (1993) "Identification of protein coding regions by database similarity search." Nature Genet. 3:266_272; Madden, T.L., Tatusov, R.L. & Zhang, J. (1996) "Applications of network BLAST server" Meth. Enzymol. 266:131_141; Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. (1997) "Gapped BLAST and PSI_BLAST: a new generation of protein database search programs." Nucleic Acids Res. 25:3389_3402; Zhang, J. & Madden, T.L. (1997) "PowerBLAST: A new network BLAST application for interactive or automated sequence analysis and annotation." Genome Res. 7:649_656.
- 15 Preferably about: 1, 2, 3, 4, 5, 6 to 10, 10 to 25, 26 to 50 or 51 to 100, or 101 to 250 nucleotides or amino acids are modified. The invention includes polypeptides with mutations that cause an amino acid change in a portion of the polypeptide not involved in providing activity or an amino acid change in a portion of the polypeptide involved in providing activity so that the mutation increases or
- 20 decreases the activity of the polypeptide.

Screening for ACE2 activators

- Small organic molecules are screened to determine if they increase ACE2 expression or activity. Polypeptide fragments of ACE2 as well as polypeptides having sequence identity to ACE2 are also tested to determine if they increase
- 25 ACE2 activity in vitro assays and in vivo in cell lines. Activators are preferably directed towards specific domains of ACE2 to increase ACE2 activation. To achieve specificity, inhibitors should target the unique sequences of ACE2.

5 The present invention also includes the isolation of substances that increase ACE2 expression. In particular ligands or substances that can bind to the ACE2 gene or protein may be isolated. Biological samples and commercially available libraries may be tested for substances such as proteins that bind to a ACE2 gene or protein. For example, the amino acid sequence of a ACE2 protein may be used to probe peptide libraries while a nucleic acid sequence encoding ACE2 may be used to probe nucleic acid libraries. In addition, antibodies prepared to ACE2 may be used to isolate other peptides with affinity for ACE2. For example, labelled antibodies may be used to probe phage displays libraries or biological samples.

15 Conditions which permit the formation of complexes with a substance and a ACE2 gene or protein may be selected having regard to factors such as the nature and amounts of the substance and the ACE2 gene or protein. The substance-protein or substance-gene complex, free substance or non-complexed substance may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. To facilitate the assay of the components, antibody against ACE2 or the substance, or labelled protein, or a labelled substance may be utilized. The antibodies, proteins, or substances may be labelled, as appropriate, with a detectable substance as described below.

25 Once potential binding partners have been isolated, screening methods may be designed in order to determine if the substances that bind to the ACE2 genes or proteins are useful in the methods of the present invention to enhance ACE2 expression on a cell and therefore useful in treating disease.

Therefore, the invention also provides methods for identifying substances which are capable of binding to ACE2 genes or proteins. In particular, the methods may be used to identify substances which are capable of binding to and

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augmenting or enhancing expression of the ACE2. Accordingly the invention provides a method of identifying substances which bind with a ACE2 gene or protein comprising the steps of:

(a) reacting a ACE2 gene or protein, preferably immobilized, and
5 a test substance, under conditions which allow for formation of a complex, and

(b) assaying for complexes, for free substance, and for non-complexed gene or protein.

Any assay system or testing method that detects protein-protein interactions may be used including co-immunoprecipitation, crosslinking and co-purification
10 through gradients or chromatographic columns may be used. Additionally, x-ray crystallographic studies may be used as a means of evaluating interactions with substances and molecules. For example, purified recombinant molecules in a complex of the invention when crystallized in a suitable form are amenable to detection of intra-molecular interactions by x-ray crystallography. Spectroscopy
15 may also be used to detect interactions and in particular, Q-TOF instrumentation may be used. Biological samples and commercially available libraries may be tested for ACE2 binding peptides. In addition, antibodies prepared to the ACE2 may be used to isolate other peptides with ACE2 binding affinity. For example, labelled antibodies may be used to probe phage display libraries or biological
20 samples. In this respect peptides may be developed using a biological expression system. The use of these systems allows the production of large libraries of random peptide sequences and the screening of these libraries for peptide sequences that bind to particular proteins. Libraries may be produced by cloning synthetic DNA that encodes random peptide sequences into appropriate
25 expression vectors. (see Christian et al 1992, J. Mol. Biol. 227:711; Devlin et al, 1990 Science 249:404; Cwirla et al 1990, Proc. Natl. Acad. Sci. USA, 87:6378). Libraries may also be constructed by concurrent synthesis of overlapping

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peptides (see U.S. Pat. No. 4,708,871). Activators are tested in hypertension, heart or kidney disease model animals.

In one embodiment, the invention includes an assay for evaluating whether a candidate compound is capable of increasing ACE2 expression or activity by
5 culturing cells (preferably kidney or heart cells) in the presence of at least one compound whose ability to activate expression or activity is sought to be determined and thereafter monitoring the cells for an increase in the level of ACE2 expression and/or activity. Increased ACE2 expression and/or activity indicates that the candidate compound is useful for treating heart or kidney
10 disease or hypertension.

A similar screening assay may be done with mammals known in the art that are prone to heart disease or kidney disease. The candidate compound is administered to the mammal and ACE2 expression and/or activity is measured. Increased ACE2 expression and/or activity indicates that the candidate
15 compound is useful for treating heart or kidney disease.

A method of determining whether a candidate compound increases the activity of ACE2 (and is useful for treating heart disease and/or kidney disease and/or hypertension) can also include:

- a) contacting (i) ACE2, a fragment of ACE2 or a derivative of either of the
20 foregoing with (ii) an ACE2 substrate in the presence of the candidate compound; and
- b) determining whether ACE2 activity on the substrate is increased, thereby indicating that the compound increases the activity of ACE2. Increased ACE2 activity indicates that the compound is useful for treating heart diseases or kidney
25 diseases listed in this application or hypertension. Determination of an increase in ACE2 activity preferably involves determining whether the compound increases ACE2 proteolytic activity (increased hydrolysis of substrates). In a

variation of the invention, the assays of the invention are subject to the proviso that the candidate compound is not a sodium-halogen salt or a potassium-halogen salt and the assay is not directed to measuring the effect of increasing ion concentration on ACE2 proteolysis. ACE2 substrates include AngI, AngII, des-Ang, AngII, apelin-13, dynorphin 13, beta-casomorphin and neurotensin.

Methods for producing ACE2 are described in CA 2,372,387. An example of an in vitro assay for ACE2 activation is shown in Vickers et al. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. J Biol. Chem. 2002, 277(17):14838. ACE2. Other assays (as well as variations of the above assays) will be apparent from the description of this invention and techniques such as those disclosed in U.S. Patent No. 5,851,788, 5,736,337 and 5,767,075 which are incorporated by reference in their entirety.

Knock-Out Mammals

- Working examples of the cloning of mouse ACE2 and generation of ACE2 knock-out mice are described in the examples below. The term "knockout" refers to partial or complete reduction of the expression of at least a portion of a polypeptide encoded by an ACE2 gene of a single cell, selected cells, or all of the cells of a mammal. The mammal may be a "heterozygous knockout", wherein one allele of the endogenous gene has been disrupted and one allele still exists. In ACE2 on the X chromosome, females may be heterozygous. In males, there is only one allele and males are homozygous. Alternatively, the mammal may be a "homozygous knockout" wherein both alleles of the endogenous gene have been disrupted.
- The term "knockout construct" refers to a nucleotide sequence that is designed to decrease or suppress expression of a polypeptide encoded by an endogenous gene in one or more cells of a mammal. The nucleotide sequence used as the

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knockout construct is typically comprised of (1) DNA from some portion of the endogenous gene (one or more exon sequences, intron sequences, and /or promoter sequences) to be suppressed and (2) a marker sequence used to detect the presence of the knockout construct in the cell. The knockout construct is inserted into a cell containing the endogenous gene to be knocked out. The knockout construct can then integrate within one or both alleles of the endogenous ACE2 gene, and such integration of the ACE2 knockout construct can prevent or interrupt transcription of the full-length endogenous ACE2 gene. Integration of the ACE2 knockout construct into the cellular chromosomal DNA is typically accomplished via homologous recombination (i.e., regions of the ACE2 knockout construct that are homologous or complimentary to endogenous ACE2 DNA sequences can hybridize to each other when the knockout construct is inserted into the cell; these regions can then recombine so that the knockout construct is incorporated into the corresponding position of the endogenous DNA).

Typically, the knockout construct is inserted into an undifferentiated cell termed an embryonic stem cell (ES cell). ES cells are usually derived from an embryo or blastocyst of the same species as the developing embryo into which it can be introduced, as discussed below.

The phrases "disruption of the gene", "gene disruption", "suppressing expression", and "gene suppression", refer to insertion of an ACE2 nucleotide sequence knockout construct into a homologous region of the coding region of the endogenous ACE2 gene (usually containing one or more exons) and/or the promoter region of this gene so to decrease or prevent expression of the full length ACE2 molecule in the cell. Insertion is usually accomplished by homologous recombination. By way of example, a nucleotide sequence knockout construct can be prepared by inserting a nucleotide sequence comprising an antibiotic resistance gene into a portion of an isolated nucleotide

sequence encoding ACE2 that is to be disrupted. When this knockout construct is then inserted into an embryonic stem cell ("ES cell"), the construct can integrate into the genomic DNA of at least one ACE2 allele. Thus, many progeny of the cell will no longer express ACE2 at least in some cells, or will express it at
5 a decreased level and/or in a truncated form, as at least part of the endogenous coding region of ACE2 is now disrupted by the antibiotic resistance gene.

The term "marker sequence" refers to a nucleotide sequence that is (1) used as part of a larger nucleotide sequence construct (i.e., the "knockout construct") to disrupt the expression of ACE2 and (2) used as a means to identify those cells
10 that have incorporated the ACE2 knockout construct into the chromosomal DNA. The marker sequence may be any sequence that serves these purposes, although typically it will be a sequence encoding a protein that confers a detectable trait on the cell, such as an antibiotic resistance gene or an assayable enzyme not naturally found in the cell. The marker sequence will also typically
15 contain either a homologous or heterologous promoter that regulates its expression.

Included within the scope of this invention is a mammal in which one or both ACE2 alleles, as well as one or both alleles of another gene(s), have been knocked out. Such a mammal can be generated by repeating the procedures set
20 forth herein for generating an ACE2 knockout mammal but using another gene, or by breeding two mammals, one with one or both alleles of ACE2 knocked out, and one with one or both alleles of a second gene knocked out, to each other, and screening for those offspring that have the double knockout genotype (whether a double heterozygous or a double homozygous knockout genotype, or
25 a variation thereof).

Other knock out animals and cells may be made using similar techniques.

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Pharmaceutical compositions

Activators of ACE2 expression and activity are preferably combined with other components, such as a carrier, in a pharmaceutical composition. These compositions may be administered to a subject, such as a human, in soluble form to prevent or treat heart disease, kidney disease or hypertension. Heart diseases include CHF, chronic heart failure, left ventricular hypertrophy, acute heart failure, myocardial infarction, and cardiomyopathy. Kidney disease includes kidney failure. ACE2 activators are useful for regulating blood pressure and arterial hypertension. Normal blood pressure has a diastolic blood pressure of less than 85 mm Hg. High normal blood pressure has a diastolic blood pressure between 85 and 89 mm Hg. Mild hypertension corresponds to a diastolic blood pressure between 90-104 mm Hg. Moderate hypertension has to a diastolic blood pressure between 105 and 114 mm Hg. Severe hypertension has a diastolic blood pressure higher than 115 mm Hg. Abnormal blood pressure is also determined from the systolic blood pressure (when the diastolic pressure is less than 90 mm Hg). Normal blood pressure has to a systolic blood pressure of less than 140 mm Hg. Borderline systolic hypertension shows a systolic blood pressure between 140 and 159 mm Hg. Isolated systolic hypertension has a systolic blood pressure higher than 160 mm Hg. (Cecil: Essentials of Medicine, Third Edition by Andreoli et al. W.B. Saunders Company (1993)). Hypertension is diagnosed in an adult over 18 years old if the average of two or more blood pressure measurements on at least two visits is 90 mm Hg or higher diastolic or 140 mm Hg systolic. Children and pregnant women have a lower blood pressure, so a blood pressure over 120/80 (i.e., 120 mm Hg systolic blood pressure/80 mm Hg diastolic blood pressure) indicates hypertension.

The pharmaceutical compositions can be administered to humans or animals by a variety of methods including, but not restricted to topical administration, oral administration, aerosol administration, intratracheal instillation, intraperitoneal

injection, and intravenous injection. Dosages to be administered depend on patient needs, on the desired effect and on the chosen route of administration. Polypeptides may be introduced into cells using *in vivo* delivery vehicles such as but not exclusive liposomes.

- 5 The pharmaceutical compositions can be prepared by known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, such that an effective quantity of the nucleic acid molecule or polypeptide is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA).

- 15 On this basis, the pharmaceutical compositions could include an active compound or substance, such as a nucleic acid molecule or polypeptide, in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and isoosmotic with the physiological fluids. The methods of combining the active molecules with the vehicles or combining them with diluents is well known to those skilled in the art. The composition could include a targeting agent for the transport of the active compound to specified sites within tissue

20 Heterologous overexpression of ACE2

- Expression vectors are useful to provide high levels of ACE2 expression. Cell cultures transformed with the nucleic acid molecules of the invention are useful as research tools, particularly for studies of ACE2 decreased states. The invention includes vectors selective for heart cells and kidney cells preferably endothelial cells which normally make ACE2. The invention also includes transfected cells including these vectors. Examples of vectors for heart and kidney cells are described, for example, in Rosengart et al. US Patent No.

- 6,322,536; March et al. US Patent No. 6,224,584; Hammond et al. US Patent No. 6,174,871; Wolfgang-M. Franz et al. Analysis of tissue-specific gene delivery by recombinant adenoviruses containing cardiac-specific promoters. Cardiovascular Research 35(1997) 560-566; Rothmann T. et al. Heart muscle-specific gene expression using replication defective recombinant adenovirus. Gene Ther 1996 Oct;3(10):919-26; Phillips MI et al. Vigilant vector: heart-specific promoter in an adeno-associated virus vector for cardioprotection. Hypertension 2002, Feb; 39(2 Pt 2):651-5; Herold BC et al. Herpes simplex virus as a model vector system for gene therapy in renal disease. Kidney Int 2002 Jan;61 Suppl 1:3-8; Figlin RA et al. Technology evaluation: interleukin-2 gene therapy for the treatment of renal cell carcinoma. Curr Opin Mol Ther 1999 Apr;1(2):271-8; Varda-Bloom N et al. Tissue-specific gene therapy directed to tumor angiogenesis. Gene Ther 2001 Jun;8(11):819-27; Scott-Taylor TH et al. Adenovirus facilitated infection of human cells with ecotropic retrovirus. Gene Ther 1998 May;5(5):621-9; Langer JC et al. Adeno-associated virus gene transfer into renal cells: potential for in vivo gene delivery. Exp Nephrol 1998 May-Jun;6(3):189-94; Lien YH et al. Gene therapy for renal diseases. Kidney Int Suppl 1997 Oct;61:S85-8; Ohno K et al. Cell-specific targeting of Sindbis virus vectors displaying IgG-binding domains of protein A. Nat Biotechnol 1997 Aug;15(8):763-7.
- 20 Cell cultures, preferably heart and kidney cell cultures and endothelial cell cultures, are used in overexpression and research according to numerous techniques known in the art. For example, a cell line (either an immortalized cell culture or a primary cell culture) may be transfected with a vector containing a ACE2 nucleic acid molecule (or molecule having sequence identity) to measure
- 25 levels of expression of the nucleic acid molecule and the activity of the nucleic acid molecule and polypeptide. The cells are also useful to identify compounds that bind to and activate the polypeptide.

Diagnostic Kits Measuring ACE2 Activity and/or Expression

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The measurement of ACE2 expression or activity is also used in: i) diagnosis of heart or kidney disease and/or hypertension ii) identifying patients at risk of developing such disease prior to the development of disease iii) measuring therapeutic response in patients having heart disease such as coronary artery disease, chronic heart failure, or kidney disease and/or hypertension and iv).
5 measuring the success of interventional disease preventive strategies in such patients at risk. The invention includes a method for assessing the levels ACE2 in a subject comprising the following steps: (a) preparing a heart or kidney sample from a specimen collected from the subject; (b) testing for the presence
10 of ACE2 in the sample; and (c) correlating the presence or levels of ACE2 in the sample with the presence (or risk) of disease such as heart or kidney disease in the subject. ACE2 levels below normal or low ACE2 activity indicate the presence or risk of disease.

Diagnostic Kits based on ACE2 single nucleotide polymorphisms

15 This invention also shows that decreased human ACE2 expression results from polymorphisms that control ACE2 gene expression. The QTL mappings in the rats show that there is a 100% correlation between reduced expression levels of ACE2 and hypertension and cardiovascular and kidney disease. None of the polymorphisms described below are found within the ACE2 coding region. All
20 are upstream or downstream of the ACE2 coding region. None of these polymorphisms or their role in heart disease, kidney disease and hypertension were previously known. A particular SNP haplotype is associated with increased risk of disease. This haplotype is an important diagnostic tool for the assessment of risk of disease and for the determination of appropriate medical treatment.

25 The polymorphisms and their nucleotide nos. are as follows:

SNP name	SNP description	Nucleotide No.	African Am	Asian	Caucasian	Reference
ACE2a rs879922	C(C/G)		60	100	70	C

ACE2b	T(C/T)	100	100	70	T
rs757066					
ACE2c	C(C/G)	70	50	80	C
rs714205					
ACE2d	C(A/C)	50	90	90	A/C
rs329442					
ACE2e	C(C/T)	80	100	60	C
rs233574					
ACE2f	C(C/T)	90	100	50	C
rs1978124					
ACE2g	A(A/G)	70	100	100	A
rs1514282					
ACE2h	A(A/G)	20	50	30	A
rs1514282-2					
ACE2i	A(A/G)	70	100	100	A
rs1514281					
ACE2j	A(A/G)	20	50	50	A
rs1514281-2					
ACE2k	A(A/G)		100	70	A
rs1514279		Failed			
ACEI	C(C/T)	80	100	80	C
2 rs1514280					
ACE2m	C(C/T)	100	100	50	C
rs233575					

The chart shows the percentage of the reference base found in each of the three populations in the chart (African American, Caucasian, Asian). For example, for SNP rs233574, the predicted SNP is C/T, with the reference peak being C. In this case, the African American allele frequency is 80% C; the Asian allele frequency is 100% C (in other words, a monomorphic marker); and the Caucasian allele frequency is 60% C.

The present invention provides polynucleotide probes which can be used to determine a subject's genotype which is whether a person is homozygous for one or the other of the polymorphisms, or heterozygous for these polymorphisms, and by extension, the person's phenotype. The phenotype indicates the amount of ACE2 expression in the person's cells. Further, the invention provides methods of using such polynucleotides in such genotype and phenotype determinations. The oligonucleotides of the invention can be used as probes to

detect nucleic acid molecules according to techniques known in the art (for example, see US patent nos. 5,792,851 and 5,851,788).

For example, a polynucleotide of the invention may be converted to a probe by being end-labelled using digoxigenin-11-deoxyuridine triphosphate. Such probes
5 may be detected immunologically using alkaline-phosphate-conjugated polyclonal sheep antidigoxigenin F(ab) fragments and nitro blue tetrazolium with 5-bromo-4-chloro-3-indoyl phosphate as chromogenic substrate.

Thus, in accordance with one aspect of the present invention, a polynucleotide probe is provided that selectively hybridizes to a portion of the ACE upstream or
10 downstream sequence. A probe may be designed to hybridise to one ACE2 polymorphism under stringent conditions but not the other polymorphisms in order to distinguish a particular polymorphism.

The polymorphism-specific polynucleotide hybridization probes of the invention may comprise, for example, genomic DNA or synthetic DNA. Such
15 oligonucleotide probes can be synthesised by automated synthesis and will preferably contain about 10 - 30 bases, although as understood in the oligonucleotide probe hybridization assay art, as few as 8 and as many as about 50 nucleotides may be useful, depending on the position within the probe where the potential mismatch with the target is located, the extent to which a label on
20 the probe might interfere with hybridization, and the physical conditions (e.g., temperature, pH, ionic strength) under which the hybridization of probe with target is carried out. In accordance with conventional procedures, the design of a polynucleotide probe according to the present invention preferably involves adjusting probe length to accommodate hybridization conditions (temperature,
25 ionic strength, exposure time) while assuring polymorphism-specificity.

In accordance with another aspect of the present invention, a test kit for genotyping is provided comprising:

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(a) means for amplifying nucleic acid that comprises at least a portion of an ACE2 5' or 3' region, wherein the portion includes a nucleotide corresponding to one of ACE2a-ACE2m.

(b) a polynucleotide probe of the invention, that distinguishes one ACE2 polymorphism from the other.

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The "means for amplifying" will, as the skilled will readily understand, depend on the amplification method to be used. Thus, for example, these means might include suitable primers, a suitable DNA polymerase, and the four 2'-deoxyribonucleoside triphosphates (dA, dC, dG, dT), if amplification is to be by the PCR method. To cite another example, if the amplification is to be by a method relying on transcription, such as the 3SR method, the means will include two primers, at least one of which, when made double-stranded, will provide a promoter, an RNA polymerase capable of transcribing from that promoter, a reverse transcriptase to function in primer-initiated, DNA-directed and RNA-directed, DNA polymerization and possibly also in RNase H degradation of RNA to free DNA strands from RNA/RNA hybrids, the four ribonucleoside triphosphates (A, C, G and U), and the four 2'-deoxyribonucleoside triphosphates. In another example, if the amplification is by the ligase chain reaction, the means will include two oligonucleotides (DNAs) and a suitable DNA ligase that will join the two if a target, to which both can hybridize adjnt to one another in ligatable orientation, is present.

The oligonucleotide probes of the invention will preferably be labelled. The label may be any of the various labels available in the art for such probes, including, but not limited to ³²P; ³⁵S; biotin (to which a signal generating moiety, bound to or complexed with avidin can be complexed); a fluorescent moiety; an enzyme such as alkaline phosphatase (which is capable of catalysing a chromogenic reaction); digoxigenin, as described above; or the like.

RFLP analysis, electrophoretic SSCP analysis or sequencing analysis may also be used to detect an ACE2 polymorphism.

There has also been provided, in accordance with another aspect of the present invention, a method of typing for an ACE2 polymorphism-specific target
5 sequence in a ACE2 nucleic acid derived from a subject, comprising the steps of,

- 10 (a) obtaining, by a target nucleic acid amplification process applied to DNA from heart or kidney, an assayable quantity of amplified nucleic acid with a sequence that is that of a subsequence (or the complement of a subsequence) of an upstream or downstream region of ACE2, said subsequence including a nucleotide where an ACE2 polymorphism may occur; and
- 15 (b) analyzing (e.g., in a nucleic acid probe hybridization assay employing a polynucleotide probe according to the invention) the amplified nucleic acid obtained in step (a) to determine the base or bases at the polymorphism position.

In one application of the typing methods of the invention, the methods are applied to an individual to determine whether the individual is at risk of developing heart or kidney disease.

20 People with coronary artery disease and/or following bypass surgery, have cardiac hypoxia. It is also known as cardiac stunning or cardiac hibernation. These patients display little structural changes in the heart but have reduced heart function. It is very uncommon to have altered heart function in the absence of structural changes. In mouse models of cardiac stunning or hypoxia, the animals have a phenotype that precisely resemble that of ACE2 mice. In
25 addition, we have shown that markers of hypoxia are induced in the ACE2 deficient mice. Taken together our data show that these mice have reduced heart function due to chronic hypoxia, and thus are models of coronary artery disease.

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Thus, the polymorphisms and/or reduced ACE2 expression or activity may be used to diagnose this state in humans. Increasing ACE2 expression and activity may be used to treat this state.

Characterization of ACE2 as a Negative Regulator of the RAS

- 5 ACE2 maps to a QTL associated with hypertension in three rat models of high blood pressure and ACE2 levels are reduced in all of these hypertensive rat strains. In mice, genetic inactivation of ACE2 using homologous recombination results in increased AngII peptide levels in tissues, upregulation of hypoxia genes in heart, and severe cardiac dysfunction. Ablation of ACE expression on an ace2-
- 10 deficient background completely abolished the heart failure phenotype of ace2 single knockout mice. These data provide a new paradigm for the regulation of the renin-angiotensin system and identify ACE2 as a negative regulator of the RAS that controls heart function.

ACE2 and blood pressure control.

- 15 Most cardiovascular diseases are multifactorial quantitative traits controlled by both genetic and environmental factors³. One major factor for cardiovascular disease is the renin-angiotensin system (RAS). In contrast to ACE which is ubiquitously expressed¹⁶, the recently identified ACE2 displays tissue-specific expression. ACE2 regulates endogenous AngII levels, by competing with ACE for
- 20 its AngI substrate and/or by cleaving AngII to generate Ang1-7. Prior to this invention, nothing was known about the *in vivo* role of ACE2 in the cardiovascular system. ACE2 regulates endogenous levels of AngII. It also functions as a negative regulator of the RAS.

- In three different rat strains that develop spontaneous or diet-induced
- 25 hypertension and cardiovascular disease, ACE2 maps within a defined QTL on the X chromosome. In all of these hypertension susceptible rat strains, ACE2 mRNA and protein levels were downregulated. The SS-X locus identified in QTL

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analysis in Sabra rats was also identified as a locus that confers resistance to salt loading¹⁶. The reduction in ACE2 in the salt sensitive strain and the absence of any alteration in its expression in the resistant strain shows that ACE2 confers resistance to diet-induced blood pressure changes.

- 5 The map position and reduced expression show that ACE2 is the gene contributing to the hypertensive QTL on the X-chromosome. Moreover, increased AngI and AngII expression in *ace2* null mice confirm that ACE2 is a regulator of the RAS system *in vivo*. However, loss of ACE2 in our mice did not result in any direct changes in blood pressure even when ACE function is
- 10 blocked. Blood pressure changes only occurred when extreme cardiac dysfunction was present in older male mice. The genetic factors that contribute to hypertension do not by themselves alter blood pressure. Rather, these QTL define single determinants of blood pressure which in concert with other genetic polymorphisms promote the change in blood pressure. We identify the
- 15 association of ACE2 polymorphisms with high blood pressure in the human population. Moreover, since *ace2* maps to the X-chromosome. Importantly, our data shows that ACE2 functions as a negative regulator of increased blood pressure.

ACE2 and the control of heart function.

- 20 Unexpectedly, loss of ACE2 in mice results in profound contractile dysfunction leading to severe reduction of systemic blood pressure in older mice. Importantly, this cardiac dysfunction is completely reversed by the disruption of ACE suggesting that a catalytic product of ACE triggers contractile impairment in the absence of ACE2. Since these contractility defects can occur in the absence of
- 25 hypertrophy or any detectable changes in blood pressure, our data also provide genetic proof that the RAS regulated heart disease phenotype can be genetically uncoupled from its effects on blood pressure and cardiac hypertrophy.

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ACE inhibitors and AngII receptor blockers have been shown to have a cardioprotective role in heart failure in humans¹³, thus implicating AngII in cardiac disease. The complete abolition of the cardiac dysfunction in our *ace/ace2* double mutant mice shows that the RAS directly controls heart function and that

5 ACE2 is a critical negative regulator that antagonizes the renin-angiotensin system and heart failure. Our genetic rescue experiments strongly indicate that it is in fact a product of ACE that drives heart failure, i.e., the increase in AngII seen in the hearts of *ace2* null mice is causative for cardiac dysfunction. Whether pharmacological inhibition of the AngII receptor rescues the heart phenotypes of

10 *ace2* mutant mice needs to be determined. Interestingly, our results in flies show that mutation of the ACE homologue, ACER²⁷, results in a severe and lethal defect of heart morphogenesis (data not shown) showing that the ACE/ACE2 functions in the heart have been conserved through evolution.

The defect in the *ace2* mutant hearts is characterized by severe contractile

15 dysfunction and upregulation of hypoxia-regulated genes with only slight remodeling in older mice, no hypertrophy and no evidence of myocyte loss. These phenotypic and molecular parameters of failing hearts in *ace2* mutant mice are different from hypertrophy and dilated cardiomyopathy. Rather intriguingly, *ace2* mutant hearts resemble cardiac stunning²³ and hibernation²⁴

20 found in human cases of coronary artery disease and in cases of by-pass surgery. In these human diseases and in animal models of cardiac stunning/hibernation, chronic hypoxic conditions lead to compensatory changes in myocyte metabolism²⁴, upregulation of hypoxia-induced genes²⁸, and reduced heart function^{22-24,28}. Since ACE2 is expressed in the vascular endothelium, and

25 not cardiac myocytes¹⁵, it is likely that the effects of ACE2 are confined to the vasculature. For instance, local increases in AngII could lead to vasoconstriction resulting in hypoperfusion and hypoxia. AngII has also been shown to cause endothelial dysfunction via the induction of oxidative stress^{29,30}. The mechanisms by which loss of ACE2 can result in the upregulation of hypoxia-inducible genes

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needs to be determined. Importantly, our data show that ACE2 polymorphisms cause the pathology of coronary heart disease in humans.

EXAMPLES

ACE2 maps to a QTL on the X-chromosome in three hypertensive rat strains.

Hypertension and most cardiovascular diseases are multifactorial in nature and disease pathogenesis is influenced by multiple genetic susceptibility loci³. In various recombinant rat models, multiple QTL for hypertension have been identified. Since ACE2 maps to the X-chromosome in human¹⁴ and a QTL has been mapped to the X-chromosome in several rat models of hypertension with no candidate gene ascribed to it as yet¹⁶⁻¹⁸, ACE2 could be a candidate gene for this QTL. To facilitate chromosomal mapping of rat ACE2, the full-length rat ACE2 cDNA was cloned by screening a rat kidney cDNA library. Rat ACE2 is highly homologous to human ACE2 and is 32% identical and 42% similar to human and mouse ACE (Fig. 1a). Like human ACE2, the rat gene is comprised of a single ACE domain with a conserved zinc binding site, a signal peptide and a transmembrane domain (Fig. 1b). Similar to human, ACE2 in mouse and rat is predominantly expressed in kidney and heart, with weaker expression in lung and liver (Fig. 1c).

Radiation hybrid mapping showed that the rat ACE2 gene maps on the X-chromosome with significant LOD scores to markers *DXRat9*, *DXWox14*, *DXWox15* and *DXRat42*, placing *ace2* between *DXRat9* and *DXRat42* (Fig. 1d). Comparative mapping showed that the *ace2* map position overlaps with a QTL interval for hypertension identified in Sabra salt-sensitive rats found between markers *DXMgh12* and *DXRat8* (SS-X)¹⁶. Moreover, the chromosomal *ace2* region maps to the *BP3* QTL interval defined in stroke-prone spontaneously hypertensive rats (SHRSP) rats¹⁷, and a previously identified hypertensive BB.Xs

QTL identified on the X-chromosome of spontaneous hypertensive rats (SHR) by congenic analysis¹⁸ (Fig. 1d). Thus, ACE2 maps to a QTL on the rat X-chromosome identified in three separate models of spontaneous and diet-induced hypertension.

5 Downregulation of ACE2 expression in hypertensive rats

Since the kidney is a major site of blood pressure regulation¹⁹, ACE2 expression levels in the kidneys of these three hypertensive rat strains was determined.

ACE2 mRNA levels were initially measured in the kidneys of salt-sensitive Sabra hypertensive (SBH/y) rats and control salt-resistant Sabra normotensive (SBN/y)

10 rats. Salt loading (with DOCA-salt) had no effect on ACE2 mRNA expression in normotensive SBN/y rats. Intriguingly, in SBH/y rats, salt loading and the development of hypertension were associated with a significant reduction in ACE2 mRNA expression as compared to normotensive SBN/y rats (Fig. 2a). Of note is that ACE2 mRNA was also lower in SBH/y fed regular diet when
15 compared to SBN/y controls fed a similar diet. This latter finding is consistent with the 10-20 mmHg difference in blood pressure observed between SBH/y and SBN/y rats fed normal diet²⁰.

To measure ACE2 protein levels, an ACE2 (aa206-aa225 of mouse ACE2) specific rabbit antiserum was generated, which cross reacts with both rat and
20 human ACE2 (not shown). In line with the decreased ACE2 mRNA expression, ACE2 protein expression was markedly reduced in SBH/y animals that were fed a normal diet (Fig. 2b). Increase in blood pressure of SBH/y rats following a 4-week diet of DOCA-salt correlated with further decreased ACE2 protein expression (Fig. 2b). Salt loading did not trigger increased blood pressure²⁰ nor
25 did it alter ACE2 expression in salt-resistant SBN/y control rats (Fig. 2b). ACE2 protein levels were also significantly decreased in the kidneys of spontaneously hypertensive SHRSP and SHR animals as compared to their WKY controls (Fig. 2b). Moreover, the levels of ACE2 mRNA were markedly reduced in hypertensive

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SHRSP and SHR rats (not shown). Cloning and sequencing of the coding region of ACE2 in the hypertensive rat strains did not reveal any sequence changes, indicating that reduced ACE2 expression likely results from polymorphisms that control ACE2 gene expression. The map position and reduced expression of
5 ACE2 in three different rat strains indicate that *ace2* is a strong candidate gene for this hypertensive QTL on the X-chromosome. Moreover, reduced ACE2 expression in all three hypertensive rat strains suggested that this enzyme functions as a negative regulator.

Cloning of mouse ACE2 and generation of ACE2 knock-out mice

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- 10 To validate the candidacy of ACE2 as a QTL and to test whether ACE2 has indeed an essential role in the cardiovascular physiology and the pathogenesis of cardiovascular diseases, the mouse ACE2 gene was cloned (Fig. 1a). Similar to rat and human ACE2, murine ACE2 also maps to the X-chromosome (not shown), contains a single ACE-domain (Fig. 1b), and is predominantly expressed
15 in the kidneys and heart (Fig. 1c). Interestingly, two isoforms for ACE2 in mouse was observed in all positive tissues. Overexpression of murine ACE2 in COS cells showed that ACE2 cleaves AngI into Ang1-9 (not shown) indicating that murine ACE2 has the same biochemical specificity as human ACE2.
- 20 To determine the *in vivo* role of ACE2, the *ace2* gene in mouse was disrupted replacing exon 9 with a neomycin resistance gene effectively deleting the zinc binding catalytic domain (see Methods and Fig. 3a). Two ES-cell lines mutated at the *ace2* locus were used to generate chimeric mice, which were backcrossed to C57BL/6 to obtain germline transmission. Both mouse lines displayed identical phenotypes. Transmission of the
25 *ace2* mutation was confirmed by Southern blot analysis (Fig. 3b). The null mutation of *ace2* was verified by the absence of *ace2* mRNA transcripts and protein in Northern (not

shown) and Western blot analyses (Fig. 3c). ACE mRNA expression in the kidneys and hearts was not altered in *ace2* mutant mice (Fig. 3d).

Since the ACE2 gene maps to the X-chromosome, all male offspring were either null mutants (*ace2^{+/y}*) or wild type for ACE2 (*ace2^{+/y}*) whereas females were either wildtype (*ace2^{+/+}*), heterozygous (*ace2^{+/-}*), or homozygous (*ace2^{-/-}*) for the *ace2* mutation. It should be noted that in all experiments described below, *ace2^{+/-}* females behaved similar to *ace2^{+/+}* females and *ace2^{+/y}* males indicating that there is no apparent effect of *ace2* heterozygosity. ACE2 null mice were born at the expected Mendelian frequency, appeared healthy, and did not display any gross detectable alterations in all organs analyzed. Moreover, in contrast to *ace^{-/-}* male mice that display significantly reduced fertility, both male and female *ace2* null mice are fertile.

Normal blood pressure and kidney functions in *ace2* mutant mice

It has been previously shown that *ace*-mutant mice display reduced blood pressure and kidney pathology^{8,9}. Therefore, it was first tested whether loss of ACE2 expression affects blood pressure homeostasis and/or kidney development or function. Intriguingly, loss of ACE2 did not result in alteration of blood pressure in 3 month old *ace2^{+/y}* male (Fig. 4a) or *ace2^{-/-}* female mice (not shown) as compared to their control littermates. Since it was possible that ACE could compensate for the loss of ACE2, we treated *ace2*-deficient mice with captopril which blocks ACE but not ACE2 function^{14,15}. However, *in vivo* inhibition of ACE with captopril reduced the blood pressure of *ace^{-/-}* male mice to a similar extent as was observed in captopril treated *wild-type* littermates (Fig. 4a). Thus, even in a scenario of ACE inhibition, loss of ACE2 has no apparent direct effect on blood pressure homeostasis.

Since ACE2 is highly expressed in the kidneys, we examined kidney morphology and function. All 3 month old and 6 month old *ace2^{+/y}* male and *ace2^{-/-}* female

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mice displayed normal kidney morphology and no apparent changes in any kidney ultrastructures (Fig. 4b). Normal cellularity and kidney structures of the ductal system and glomeruli were also confirmed using serial section morphometry (not shown). Moreover, *ace2*-deficient mice displayed normal levels of creatinine, urea (BUN), and potassium in their serum (Fig. 4c) and these mice exhibited normal hematocrit and hemoglobin levels (Fig. 4d). In addition, there was no evidence of proteinuria (data not shown). Thus, in both male and female mice, loss of ACE2 does not appear to have an essential role in blood pressure homeostasis or kidney function.

10 Loss of ACE2 results in a severe defect in heart function

Pharmacological inhibition of ACE or AngII receptors suggested a role for the RAS in the regulation of heart function and cardiac hypertrophy¹³. However, neither *ace*^{8,9} nor *angiotensinogen*²¹ null mice develop any overt heart disease. Since ACE2 is highly expressed in the vasculature of the heart, hearts of *ace2*-deficient mice were analyzed. Hearts of *ace2* mutant mice display a slight wall thinning of the left ventricle and increased chamber dimensions (Fig. 5a). Thinning of the anterior left ventricular wall (AW) and increase in the left ventricle end diastolic dimension (LVEDD) in *ace2*-deficient hearts can be also seen by echocardiography (Table 1). These structural changes are primarily observed in 6 month old male mice. However, heart body weight ratios were comparable between age matched 3 month old (not shown) and 6 month old *ace2*^{+/y} and *ace2*^{+/y} mice (Fig. 5a,b). Echocardiography also showed that the left ventricle mass (LVM) and LVM/body weight ratios were normal (Table 1). Structural and biochemical changes characteristic of dilated cardiomyopathy were not observed as there was no indication of interstitial cardiac fibrosis (Fig. 5c,d) nor prototypical changes in ANF, BNP, β -MHC, α -MHC, and skeletal muscle actin gene expression (not shown). In addition, individual cardiomyocytes of ACE2 null mice exhibited no evidence of hypertrophy and we did not observe any evidence

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of altered cardiomyocyte apoptosis in ACE2 null mice as detected by TUNEL staining (not shown). Thus, despite mild dilation of hearts in 6 month old ACE2 null mice, there was no evidence of cardiac hypertrophy or dilated cardiomyopathy.

- 5 Interestingly, assessment of cardiac function by echocardiography revealed that all *ace2^{-/-}* male and *ace2^{-/-}* female mice exhibit severe contractile heart failure as determined by decreased fractional shortening (FS), and decreased velocity of circumferential fibre shortening (Table 1 and Fig. 6a,b). The decrease in function was found to be more severe in 6 month old male and female mice as compared
10 to age matched 3 month old mice, suggesting a progression in the phenotype (Table 1). Consistent with the decreased cardiac contractility, 6 month old *ace2^{-/-}* mice exhibited reduced blood pressure (Fig. 6c), a feature not found in age matched *ace2^{-/-}* females and 3 month old males, suggesting that the reduction in blood pressure may be the result of severe cardiac dysfunction and not a direct
15 effect of loss of ACE2 on systemic blood pressure. These surprising results show that ACE2 is a critical negative regulator of heart contractility.

- To confirm the echocardiographic defects in cardiac function, invasive hemodynamic measurements were performed in *ace2* null mice. Importantly, invasive hemodynamic measurements showed that both dP/dT-max and dP/dT-
20 min were markedly reduced in the *ace2* mutant mice (Table 2), indicating severe impairment of contractile heart function. Loss of ACE2 also resulted in a significant decrease in aortic and ventricular pressures consistent with the observed reductions in cardiac contractility (Table 2). Remarkably, the data establish that the defects in cardiac contractility of *ace2* mutant mice occur in the
25 absence of any overt cardiac hypertrophy and can be genetically uncoupled from alterations in blood pressure.

Upregulation of hypoxia-inducible genes in *ace2* null mice

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5 The severe contractile dysfunction and mild dilation in the absence of hypertrophy or cardiac fibrosis in *ace2* null mice resembles cardiac stunning/hibernation in humans and animal models^{22,23}. Cardiac stunning and hibernation are adaptive responses to chronic hypoxia such as coronary artery disease or following bypass surgery²⁴. Since ACE2 is highly expressed in vascular endothelial cells but contractility is controlled by cardiomyocytes, it was speculated that loss of ACE2 could result in cardiac hypoxia. Therefore changes in the expression levels of hypoxia-inducible genes such as BNIP3²⁵ and PAI-1²⁶ were analyzed by Northern blotting. In the hearts of all *ace2* null mice analyzed, mRNA expression of BNIP3 and PAI-1 were markedly upregulated as compared to their *wild-type* littermates (Fig. 7a). Thus, loss of ACE2 results in the induction of a hypoxia-regulated gene expression profile.

Increased Angiotensin II levels in tissues of *ace2* null mice

15 Since ACE2 functions as a carboxypeptidase, cleaving a single residue from AngI, to generate Ang1-9^{14,15}, and a single residue from AngII to generate Ang1-7¹⁴, it was hypothesized that ACE2 may function as a negative regulator of the RAS by competing with ACE for the substrate AngI and/or cleaving and inactivating AngII. If correct, loss of ACE2 should increase AngII levels *in vivo*. Using radioimmunoassays, AngII levels were indeed found to be significantly increased in the kidneys and hearts of *ace2* mutant mice (Fig. 7b). In addition, an increase in AngI was also observed (Fig. 7b) consistent with AngI being a substrate of ACE2 action *in vivo*. No differences in ACE mRNA levels were found in the hearts and kidneys of *ace2* mutant mice compared to controls indicating that the increased AngII tissue levels were not due to increased ACE expression (Fig. 3d). These data show that ACE2 functions as a negative regulator of the renin-angiotensin system and controlling endogenous levels of AngII.

Ablation of ACE expression in *ace2*-deficient mice rescues heart failure

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If the phenotype in the hearts of *ace2* mutant mice was due to the increase in AngII levels, then genetic ablation of ACE in combination with disruption of ACE2 may serve to reduce AngII levels and rescue the phenotype observed in the ACE2 mutant mice. To test this notion, *ace/ace2* double mutant mice were
5 generated. These double mutant mice were born at the expected Mendelian ratio and appear healthy. Blood pressure (Fig. 8a) and kidney defects (not shown) in the *ace-ace2* double null mice were similar to that of *ace* single mutant mice. Fertility of the *ace-ace2* double mutant mice was not addressed. Thus, loss of both ACE and ACE2 does not cause any apparent disease in addition to that
10 seen in *ace* single mutant mice.

Since the heart function of ACE knockout mice has not been previously reported the heart parameters in these mice were first analyzed. In *ace*^{-/-} mice, hearts are histologically normal (not shown) and no defect in heart function could be detected at 6 months of age (Fig. 8b,c). Importantly, ablation of ACE expression
15 on an *ace2* mutant background completely abolished the heart failure phenotype of *ace2* single knock-out mice (Fig. 8b,c). Moreover, using echocardiography, all heart functions of 6 month old, age matched *ace-ace2* double mutant mice were comparable to that of their *ace* single mutant and *wild type* littermates (Table 1). Restoration of heart functions occurred in both male and female *ace-ace2* double
20 mutant mice. These genetic data show that ACE expression is required and necessary to trigger contractile heart failure in the absence of ACE2. Importantly, there was also no difference in blood pressure between *ace* and *ace/ace2* knockout mice (Fig. 8a), further implying that the reduced blood pressure in older male *ace2* mice is due to the dramatic decrease in heart
25 function.

Methods

Cloning of mouse and rat ACE2 and chromosomal QTL mapping. Murine ACE2 was cloned from a proprietary EST database. Using a mouse ACE2 probe,

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we then screened a rat kidney cDNA (Invitrogen) to obtain a full-length rat cDNA as determined by DNA sequencing. For chromosomal mapping, a rat *ACE2* cDNA specific probe was used to screen a rat PAC library (RPCI-31, Research Genetics), identifying two positive clones (6M6 and 125K9). The end sequences of these clones were determined and rat specific primers were designed (mc2L: 5'-TCAATTTACTGCTGAGGGGG-3', mc2R: 5'-GAGGGATAACCCAGTGCAAA-3') to determine the chromosomal map position of *ACE2* in rat by screening a radiation hybrid panel (RH07.5, Research Genetics). SHR and control WKY rats were obtained from Harlan and maintained at the animal facilities of the Ontario Cancer Institute in accordance with institutional guidelines. Tissues from SHRSP rats were kindly provided by Dr. Detlev Ganten, Germany. Salt-resistant and salt-sensitive Sabra rats were bred and maintained at the animal facility of the Ben-Gurion University Barzilai Medical Center, Israel. Doca-salt treatment was as described previously¹⁶.

Expression analysis. Total RNA was prepared from rat kidneys using tri-reagent. 20 μ g of RNA was resolved on a 0.8% formamide gel. Blotted to nylon membrane (Amersham), and probed with a partial rat *ACE2* cDNA clone (9-1). The β -actin probe and Multiple tissue Northern blots were purchased from Clontech. For western analysis, kidneys were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 20mM EDTA, and 1% triton-X 100) supplemented with "Complete" protease inhibitor cocktail (Roche) and 1 mM Na_3VO_4 . 100 μ g of protein was resolved by SDS-PAGE on 8% tris-glycine gels. *ACE2* immunoserum was obtained from rabbits immunized with a mouse specific *ACE2* peptide DYEAEGADGYNYNRNQLIED. The serum was affinity purified with the immunizing peptide using sulpho-link kit (Pierce). A commercially available β -actin antibody was used as loading control (Santa Cruz).

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Generation of ACE2 mutant mice. A targeting vector (559 base pair short arm, and 8.1 kilobase long arm) was constructed using the pKO Scrambler NTKV-1907 vector (Stratagene). A portion of the *ace2* genomic DNA containing nucleotides +1069 to +1299 was replaced with the neomycin resistance cassette in the anti-sense orientation. The targeting construct was electroporated into E14K ES cells, and screening for positive homologous recombinant ES clones was performed by Southern blotting of EcoRI-digested genomic DNA hybridized to 5' and 3' flanking probes. Two independent *ace*^{-y} ES cell lines were injected into C57BL/6-derived blastocysts to generate chimeric mice, which were backcrossed to C57BL/6 mice. Two ES cell lines gave independent germline transmission. Data reported in this manuscript are consistent between the two mutant mouse lines. Ablation of ACE2 expression was confirmed by RT-PCR, Northern, and Western blot analyses. Only littermate mice were used for all experiments. Histology of all tissues, apoptosis assays, blood serology, and kidney morphometries were as described³¹. Complete ACE mutant mice have been previously described⁸ and were obtained from Jackson Laboratories. Mice were maintained at the animal facilities of the Ontario Cancer Institute in accordance with institutional guidelines.

Heart morphometry, echocardiography, hemodynamics and blood pressure measurements. For heart morphometry, hearts were perfused with 10% buffered formalin at 60 mmHg and subsequently embedded in paraffin. Myocardial interstitial fibrosis was determined by quantitative morphometry using the color-subtractive computer assisted image analysis using Image Processing Tool Kit version 2.5 coupled with Photoshop 6.0 software. Picro-Sirius red stained sections were used to calculate interstitial fibrosis as the ratio of the areas with positive PSR staining compared to the entire visual field. Echocardiographic assessments were performed as described³² using *wild-type* and *mutant* littermates. Mice were anesthetized with isoflurane/oxygen and examined by transthoracic echocardiography using a Acuson® Sequoia C256

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equipped with a 15MHz linear transducer. (FS) was calculated as: $FS = [(EDD - ESD)/EDD] \times 100$. Vcfc was calculated as FS/ejection time corrected for heart rate. Hemodynamics measurements were performed as described³³. Briefly, mice were anesthetized, and the right carotid artery was isolated and cannulated with
5 a 1.4 French Millar catheter (Millar Inc., Houston) connected to an amplifier (TCP-500, Millar Inc.). After insertion of the catheter into the carotid artery, the catheter was advanced into the aorta and then into the left ventricle to record the aortic and ventricular pressures. The parameters measured and analyzed were heart rate, aortic pressure, left ventricular (LV) systolic pressure, LV diastolic
10 pressure, and the maximum and minimum first derivatives of the LV pressure (+dP/dtmax and dP/dtmax, respectively). Tail-cuff blood pressure measurement were taken using a Visitech BP-2000 Blood Pressure Analysis System manufactured by Visitech Systems (Apex, NC). For captopril treatment, drinking water was supplemented with 400mg/L captopril (Sigma) for two weeks prior to
15 blood pressure measurement.

Tissue angiotensin peptide levels. Hearts and kidneys were homogenized on ice in 80% ethanol / 0.1 N HCl containing the peptidase inhibitors described above including phenylmethylsulfonyl fluoride (PMSF, 100 μ M). Protein homogenates were centrifuged at 30,000 g for 20 minutes, supernatants
20 decanted, and acidified with 1% (v/v) heptafluorobutyric acid (HFBA, Pierce, Rockford, IL). The supernatant was concentrated to 5 ml on a Savant vacuum centrifuge (Savant, Farmingdale, NY) and concentrated extracts were applied to activated Sep-Paks, washed with 0.1% HFBA, and eluted with 5 ml 80% methanol / 0.1% HFBA. Radioimmunoassay analysis of angiotensin peptide
25 content in the extracts from heart and kidney tissues was performed. The limits of detection for the Ang II and Ang I RIAs were 0.5 fmol/tube and Ang I 5 fmol/tube, respectively.

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The present invention has been described in detail and with particular reference to the preferred embodiments; however, it will be understood by one having ordinary skill in the art that changes can be made without departing from the spirit and scope thereof. For example, where the application refers to proteins, it is clear that peptides and polypeptides may often be used. Likewise, where a gene is described in the application, it is clear that nucleic acid molecules or gene fragments may often be used.

All publications (including Genbank entries), patents and patent applications are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

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We claim:

1. An isolated nucleic acid molecule encoding an ACE2 polypeptide, the nucleic acid comprising a nucleotide polymorphism upstream or downstream of the ACE2 nucleic acid coding region, wherein the polymorphism reduces ACE2 expression compared to wild type ACE2.
2. An isolated nucleic acid molecule according to claim 1 wherein the polymorphism is selected from the group consisting of at least one of ACE2a-ACE2m.
3. A method of detecting an ACE2 decreased state in a subject comprising obtaining a DNA sample from the subject and identifying a nucleic acid of claim 1 or 2 in the DNA sample.
4. A method for diagnosing a disease or a predisposition to a disease characterized by an ACE2-decreased state in a subject comprising identifying a nucleic acid of claim 1 or 2 in a DNA sample from the subject.
5. A method according to claim 3 or 4 comprising determining whether the subject is homozygous or heterozygous for the nucleotide polymorphism.
6. A method of any one of claims 3 to 5, wherein the ACE2 decreased state is associated with a heart disease or a kidney disease selected from a group consisting of hypertension, congestive heart failure, chronic heart failure, acute heart failure, myocardial infarction, atherosclerosis and renal failure.
7. A polynucleotide comprising a sequence which binds specifically to (i) a region upstream or downstream of an ACE2 nucleic coding region wherein the region is proximate to a nucleotide polymorphism that decreases ACE2 expression.
8. The polynucleotide of claim 7, comprising 8 to 10, 8 to 15, 8 to 20, 8 to 25, 25 to 50, 50 to 75, 50 to 100, 100 to 200, 200 to 500 or 500 to 1000 nucleotides.

9. The polynucleotide of claim 7 or 8, wherein the nucleic acid specifically binds proximate to one of ACE2a-ACE2m under high stringency hybridization conditions.
10. The polynucleotide of claim 9, wherein the stringent hybridization conditions comprise 0.1XSSC, 0.1% SDS at 65°C.
- 5 11. The polynucleotide of claim 7, comprising a sequence complementary to an ACE2 polymorphism.
12. The polynucleotide of claim 11, comprising a sequence selected from the group consisting of:
- 10 (a) 8-50 nucleotides of an upstream or downstream region of ACE2 which is proximate to a nucleotide polymorphism, wherein the sequence includes one of ACE2a-ACE2m and comprises all or part of one of the sequences in Figure 11.
- (b) a sequence that is complementary to a sequence specified in (a); and
- (c) a sequence having at least 70% sequence identity to a sequence in (a) or (b), wherein the sequence having identity is capable of hybridization to ACE2 under
- 15 high stringency hybridization conditions.
13. The polynucleotide of claim 7, wherein the nucleic acid is capable of use as a probe in a hybridization assay.
14. The polynucleotide of claim 13, wherein the nucleic acid sequence is detectably labeled.
- 20 15. The polynucleotide of claim 14, wherein the detectable label comprises:
- (a) a fluorogenic dye; and/or
- (b) a biotinylation modification; and/or

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(c) a radiolabel.

16. An ACE2 genotyping kit comprising a detection agent for detecting the presence of an ACE2 polymorphism in a nucleic acid sample derived from a subject.

5 17. The kit of claim 16, wherein the detection agent comprises a nucleic acid and/or a restriction enzyme.

18. The kit of claim 16, further comprising a biological sample container for holding the detection agent.

10 19. The kit of claim 16, further comprising a plate having a plurality of wells and having bound thereto probes having a nucleic acid sequence which specifically binds to an ACE2 sequence including an ACE2 polymorphism.

20. The kit of claim 16, further comprising an amplification agent for amplifying the nucleic acid.

15 21. The kit of claim 20, wherein the amplification agent amplifies a region of ACE2 nucleic acid proximate to an ACE2 single nucleotide polymorphism selected from the group of ACE2a-ACE2m.

22. The kit of claim 20, wherein the amplification agent comprises a primer set, wherein each primer is a nucleic acid that will specifically bind proximate to, and/or cause elongation through, one of ACE2a-ACE2m.

20 23. The kit of claim 16, for detecting that the subject has or is at risk of an ACE2 decreased state disease.

24. The kit of claim 23, wherein the disease comprises heart disease or kidney disease.

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25. The kit of claim 24, wherein the disease is selected from the group consisting of hypertension, congestive and dilative chronic heart failure, acute heart failure, myocardial infarction, coronary artery disease, atherosclerosis, and renal failure.
- 5 26. A method of ACE2 genotyping a subject comprising:
- (a) obtaining an ACE2 nucleic acid sample derived from the subject including regions upstream and downstream of the ACE2 coding region; and
- (b) detecting a region of an ACE2 nucleic acid that includes an ACE2 single nucleotide polymorphism.
- 10 27. The method of claim 26, wherein the nucleotide polymorphism is selected from the group consisting of ACE2a-ACE2m.
28. The method of claim 27, comprising determining whether the subject is homozygous or heterozygous for the ACE2 polymorphism.
29. The method of claim 28, wherein the subject is a human and the ACE2
- 15 genotype is used to determine if that the subject has, or is at risk of an ACE2 decreased state disease.
30. The method of claim 29, wherein the disease comprises a heart disease or kidney disease.
31. The method of claim 26, wherein the nucleic acid is obtained by amplifying
- 20 the nucleic acid from the subject.
32. The method of claim 31, wherein the nucleic acid is obtained by amplification with all or part of the polynucleotide of any of claims 7 to 15.
33. The method of claim 26, wherein the detection step comprises determining the nucleotide sequence of the ACE2 nucleic acid.

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34. The method of claim 26, wherein the detection step comprises contacting the nucleic acid with the polynucleotide of any of claims 7 to 15 under high stringency conditions.

5 35. The method of claim 34, wherein the polynucleotide will selectively hybridize proximate to (i) a region of ACE2 nucleic acid that includes a single polymorphism distinctive of an ACE2 polymorphism.blastblast

36. The method of claim 26, wherein the detecting step comprises:

(a) performing a restriction endonuclease digestion of the nucleic acid, thereby providing a nucleic acid digest; and

10 (b) contacting the digest with the polynucleotide.

37. The method of claim 36, wherein the hybridization occurs either during or subsequent to PCR amplification and the analysis is by "Real-Time" PCR analysis, or fluorimetric analysis.

15 38. The method of claim 36, wherein the detecting step includes size analysis of the nucleic acid .

39. A method for screening a compound that is an agonist of ACE2 activity, comprising:

a) providing: i) a purified preparation comprising ACE2, ii) a substrate, and iii) a test compound;

20 b) mixing said ACE2 and said substrate under conditions such that said ACE2 can act on said substrate to produce a product, wherein said mixing is done in the presence and absence of said test compound; and

c) measuring directly or indirectly the amount of said product produced in the presence or absence of said test compound.

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40. The method of claim 39, wherein said substrate is angiotensin I or angiotensin II.

41. The method of claim 39 wherein said product comprises Ang1-9 or Ang1-7.

42. A compound isolated according to claims 39 to 41.

5 43. A method of treating an ACE2 decreased state, comprising administering to a mammal having that condition a therapeutically effective amount of an ACE2 agonist.

44. A method according to claim 43 in which the mammal is a human.

10 45. A method of any one of claims 43 to 44, wherein the decreased ACE2 state is associated with a disorder selected from a group consisting of hypertension, congestive heart failure, chronic heart failure, acute heart failure, myocardial infarction, atherosclerosis and renal failure.

46. A method for gene therapy for an ACE2-decreased state, comprising delivering an effective amount of a transgene coding to an organ.

15 47. A method according to claim 46, wherein the affected organ is the heart or kidney.

48. The method of any one of claims 46 or 47, wherein the ACE2 transgene is administered to the patient in a gene therapy vector.

20 49. The method of claim 48, wherein the gene therapy vector comprises a viral vector.

50. The method of any one of claims 46-49, wherein the patient is a human.

51. The method of any one of claims 46-50, wherein the ACE2-decreased state is associated with a disorder selected from a group consisting of hypertension,

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congestive heart failure, chronic heart failure, acute heart failure, myocardial infarction, atherosclerosis and renal failure.

52. A non-human mammal comprising the gene ACE2 wherein one allele of the gene has been disrupted.

5 53. A non-human mammal comprising the gene encoding ACE2 wherein both alleles of the gene have been disrupted.

54. A non-human mammal comprising a disrupted ACE2 mutation, wherein the disruption results in a null mutation of the gene encoding ACE2.

55. The non-human mammal of any of claims 52 to 54 which is a rodent.

10 56. The non-human mammal of claim 55 which is a mouse.

57. The non-human mammal of claims 52 to 56 characterized by hypertension or cardiac contractility defect.

58. A nucleic acid comprising an ACE2 knockout construct.

59. A vector comprising the nucleic acid of claim 58.

15 60. A murine embryonic stem cell line comprising the nucleic acid of claim 59.

61. A method of screening compounds that modulate hypertension and cardiac contractility comprising introducing the compounds into the non-human mammal of any of claims 52 to 56 and determining the increase or decrease in blood pressure and/or cardiac contractility.

20 62. The use of an ACE2 activator as a pharmaceutical substance.

63. The use of an ACE2 activator for treatment of heart disease or kidney disease.

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64. The use of an ACE2 activator for preparation of a medicament for treatment of heart disease or kidney disease.

65. A method of medical treatment of heart disease or kidney disease in a mammal, comprising administering to the mammal in need of treatment an
5 effective amount of an ACE2 activator.

66. The method of claim 65, wherein the ACE2 activator is co-administered with an ACE inhibitor.

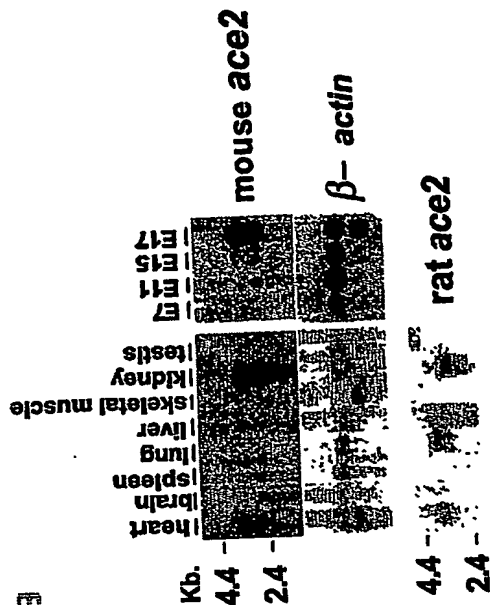
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ABSTRACT OF THE DISCLOSURE

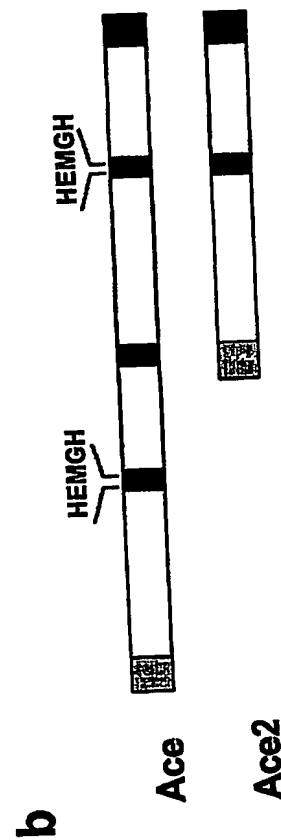
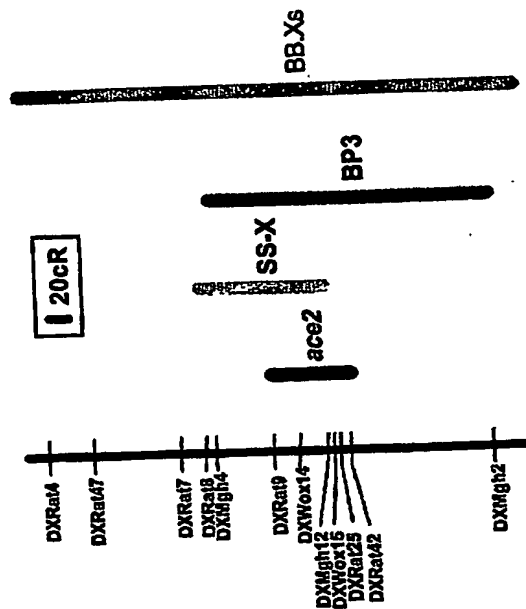
The invention relates to ACE2 activating compounds for prevention and treatment of heart disease, kidney disease and hypertension. The invention also includes methods of diagnosing heart disease, kidney disease and hypertension by measuring ACE2 expression or nucleotide polymorphism analysis.

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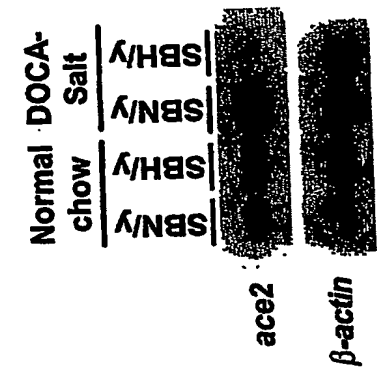


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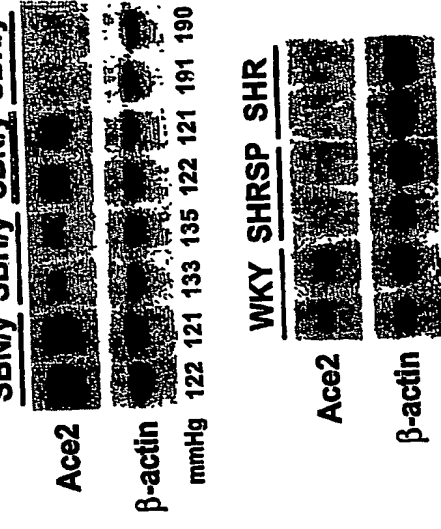


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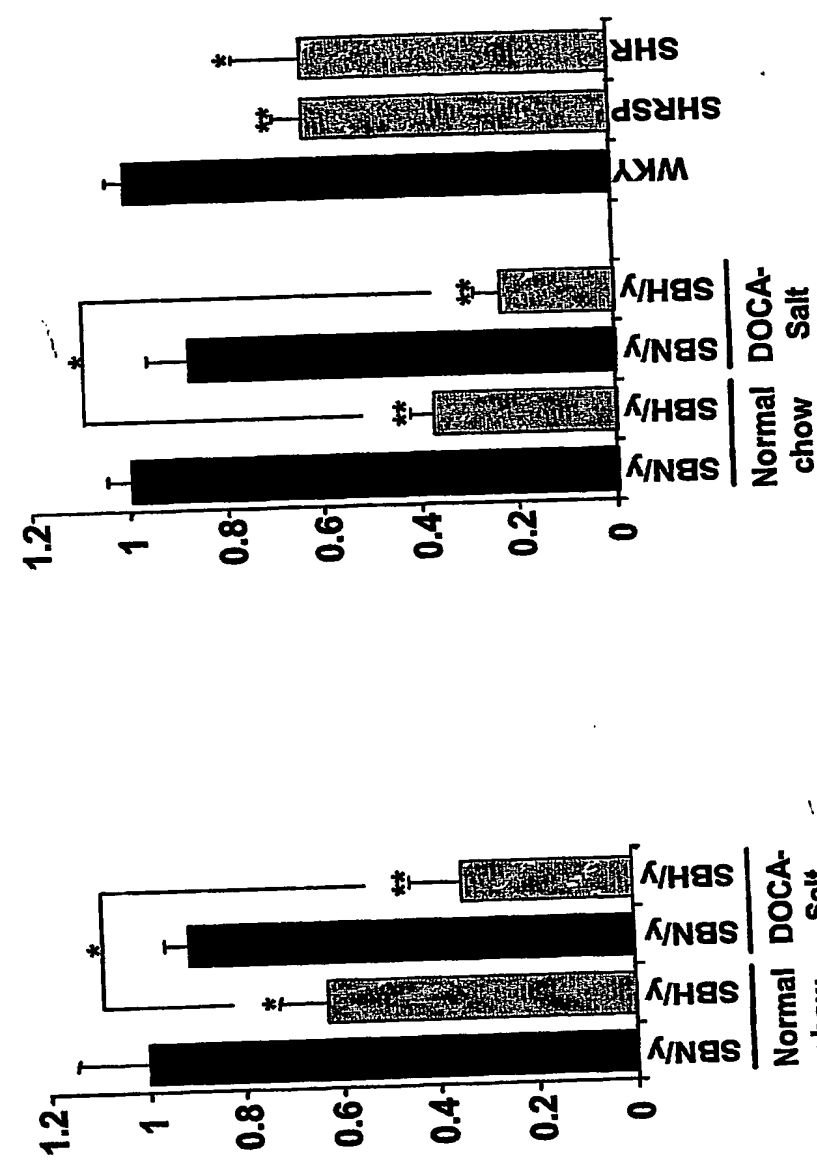
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a

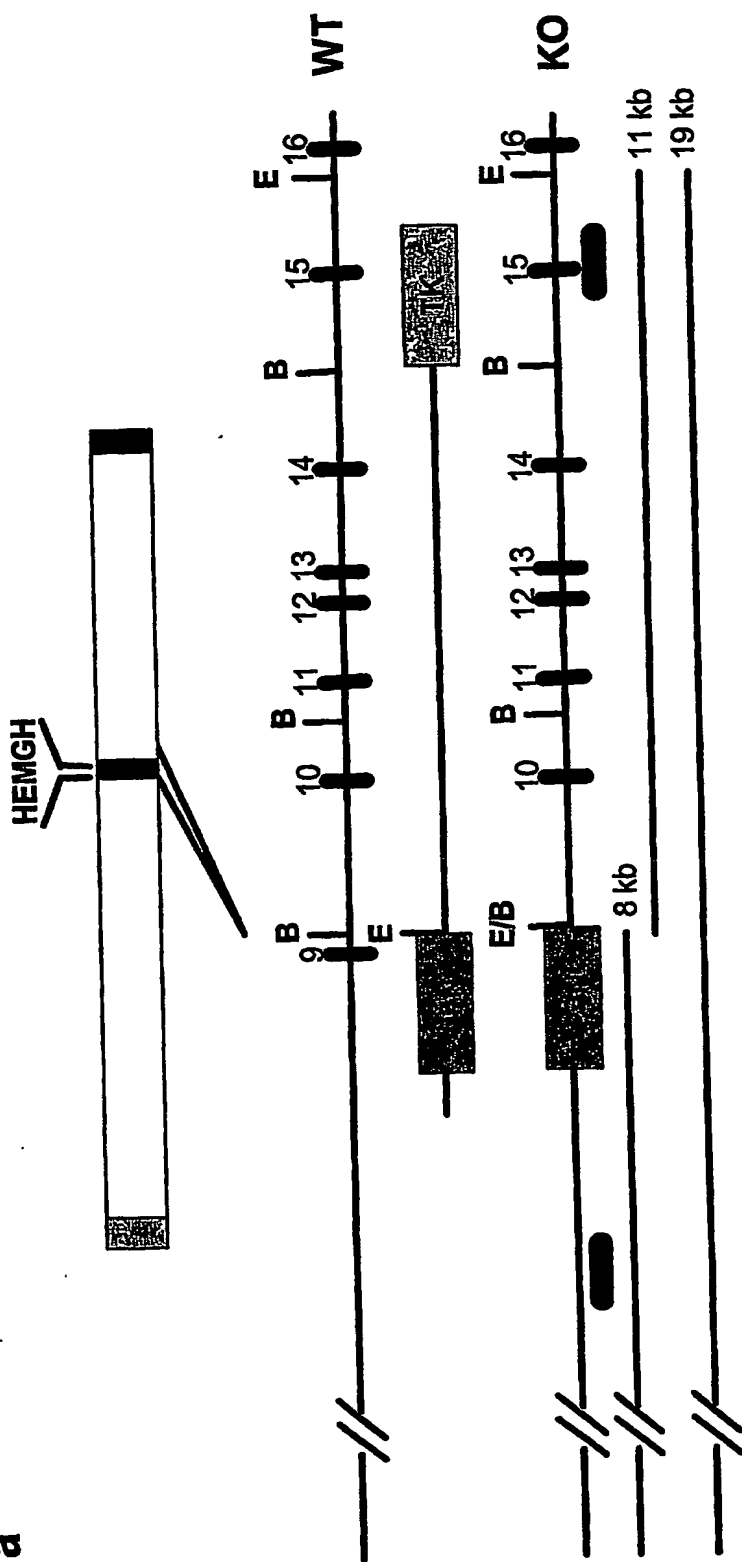


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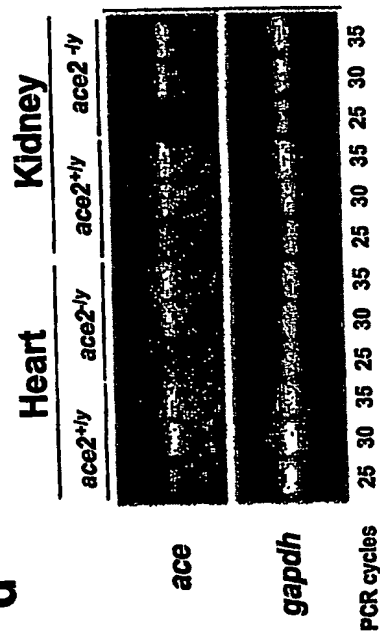


Crackower Figure 2

a



d

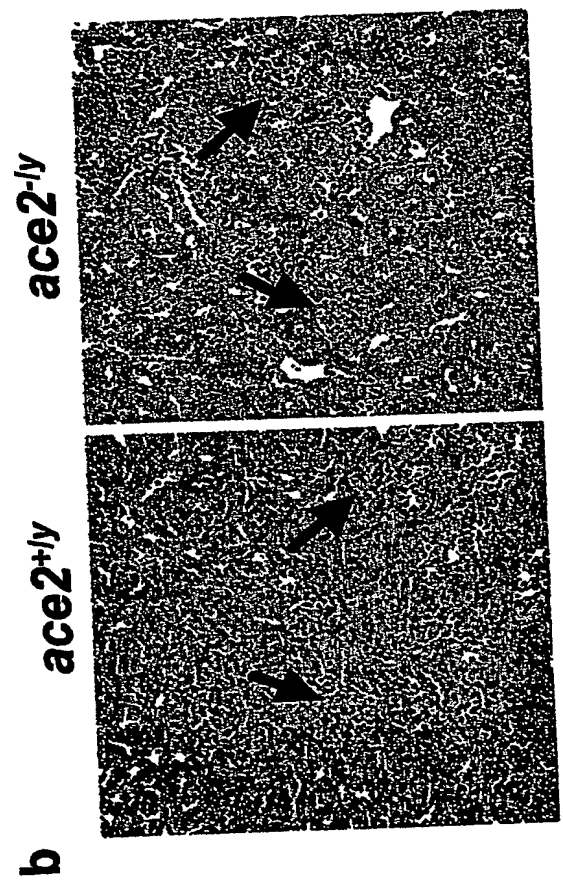
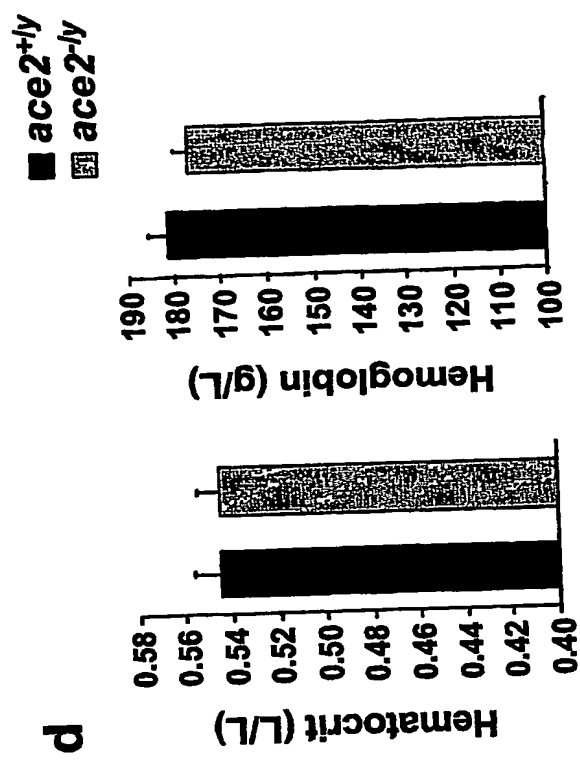
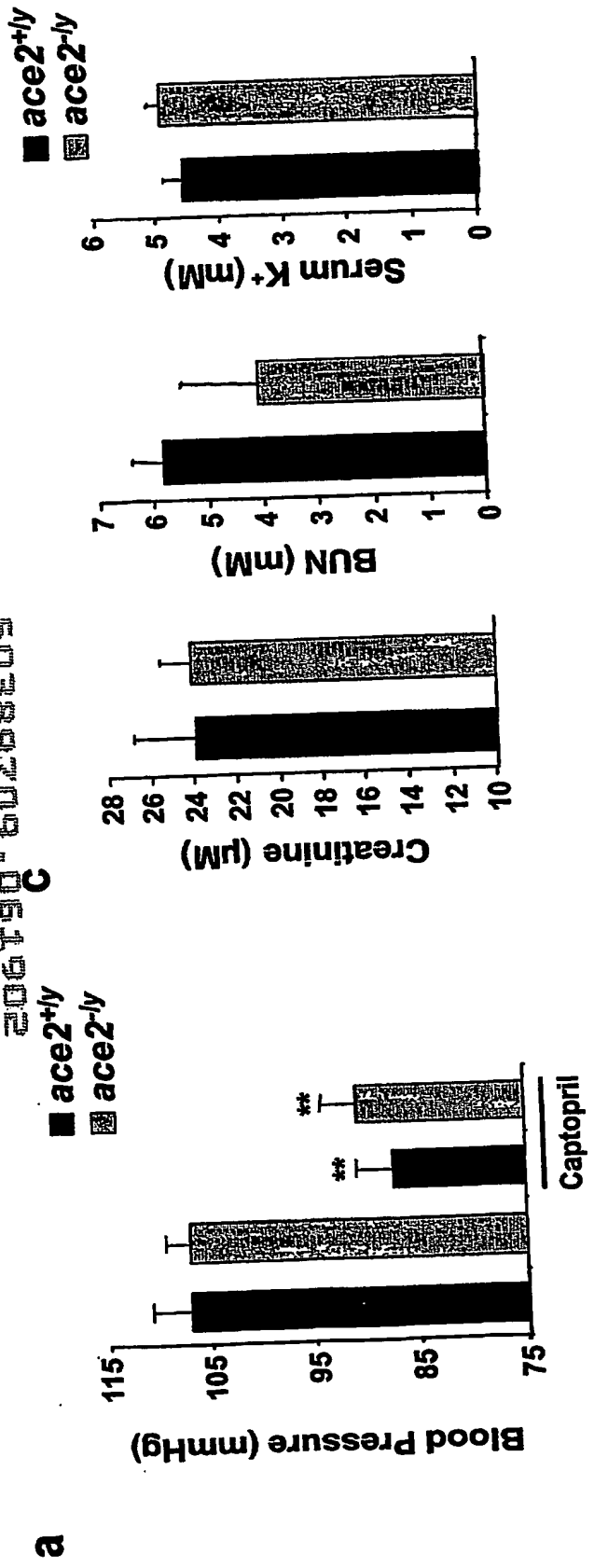


c



Crackower Figure 3

206T90-60268E09

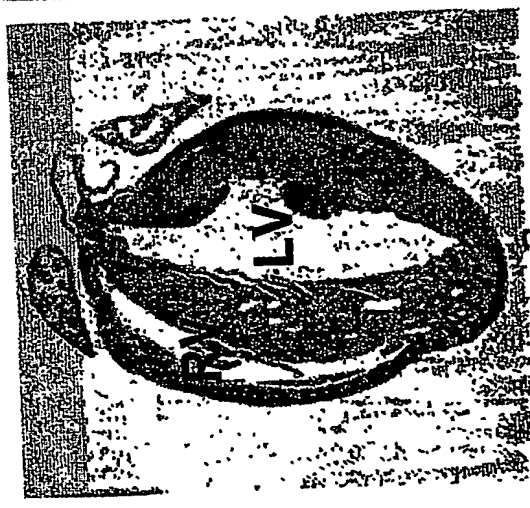


Crackower Figure 4

206T90-60268E09

a

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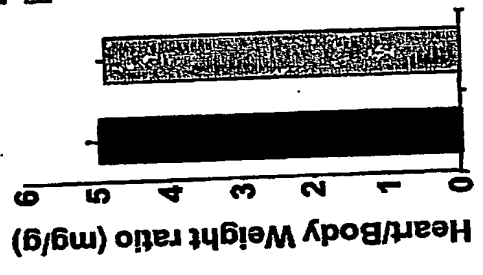


ace2^{-/-}



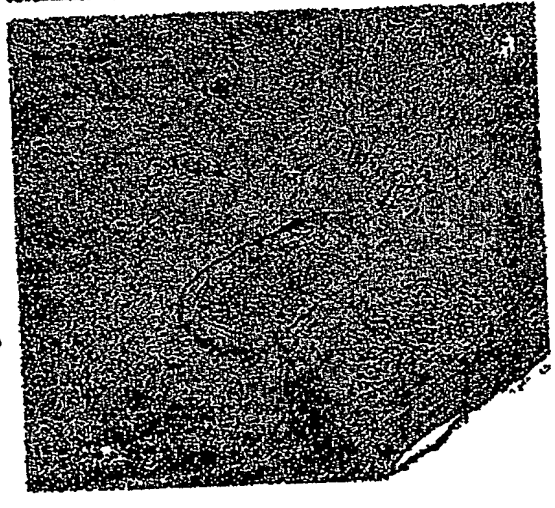
b

■ ace2^{+/-}
▨ ace2^{-/-}

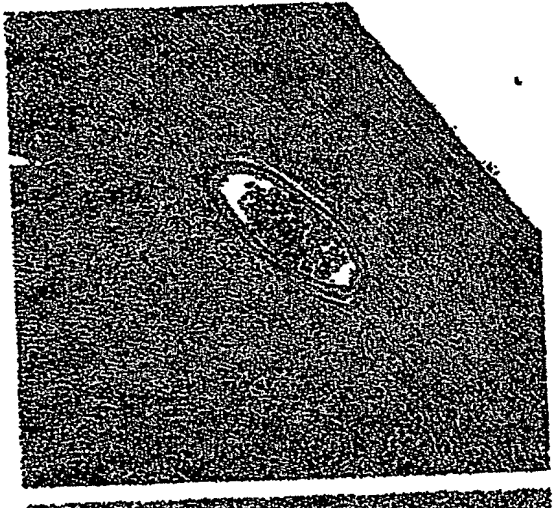


c

ace2^{+/-}

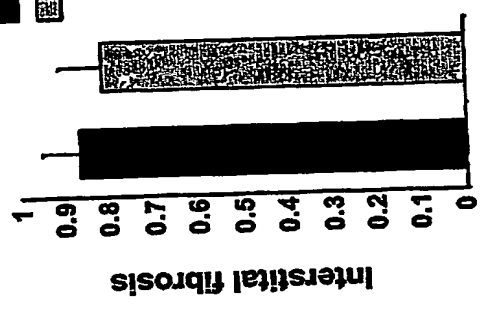


ace2^{-/-}



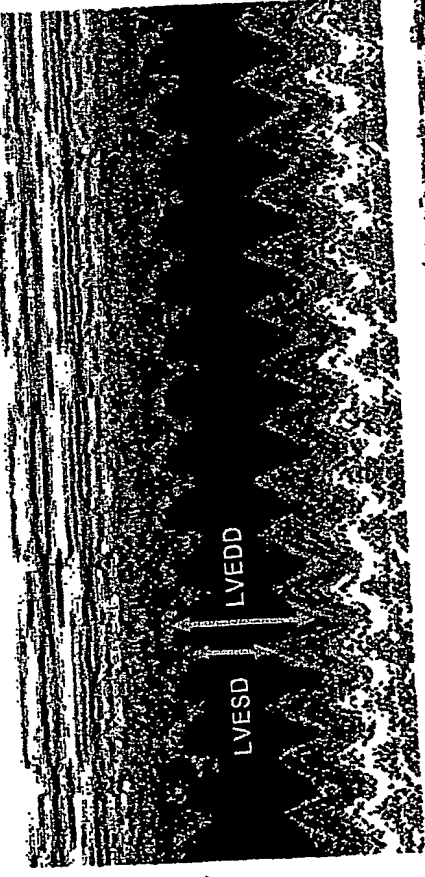
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▨ ace2^{-/-}

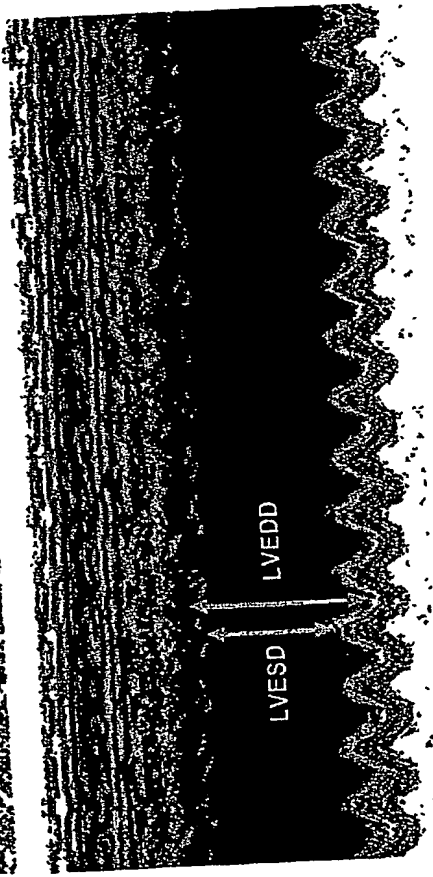


Crackower Figure 5

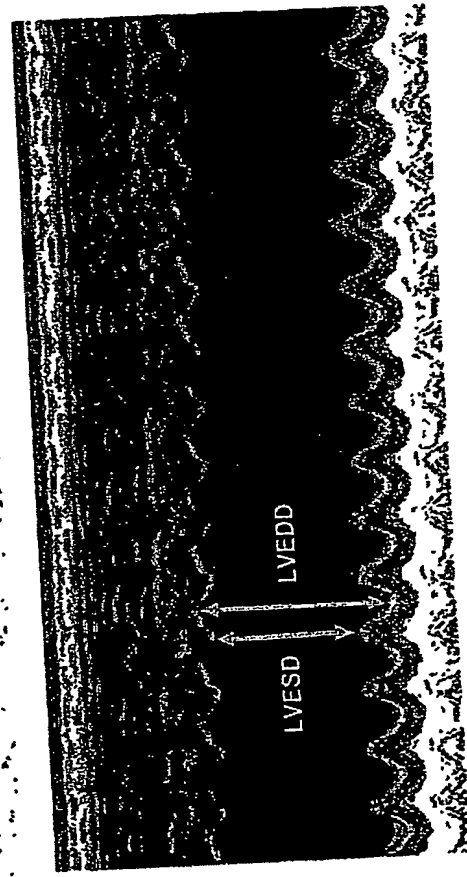
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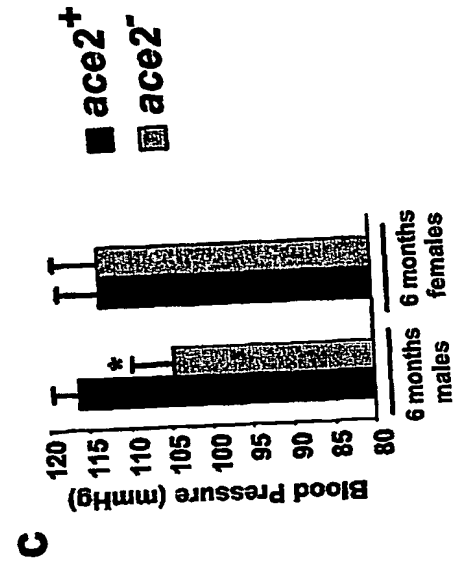
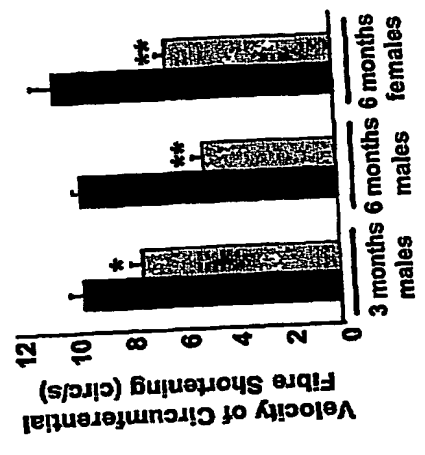
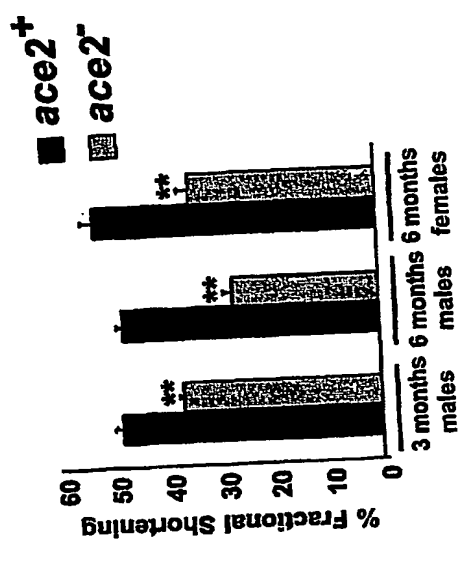
ace2^{+/ly}



ace2^{-ly}



ace2^{-ly}



Crackower Figure 6

Table 1. Heart functions of ace2 null mice

	3 month - males		6 month - males		6 month - females	
	ace2 ^{+/y}	ace2 ^{-y}	ace2 ^{+/y}	ace2 ^{-y}	ace2 ^{+/y}	ace2 ^{-y}
	n=7	n=7	n=8	n=8	n=3	n=3
Heart Rate, bpm	469 ±12	466 ±18	495 ±15	482 ±12	460 ±6	452 ±14
AW, mm	0.65 ±0.02	0.62 ±0.01	0.66 ±0.01	0.59 ±0.02*	0.65 ±0.02	0.57 ±0.04
LVEDD, mm	4.09 ±0.04	4.25 ±0.10	4.12 ±0.10	4.49 ±0.12*	3.71 ±0.10	4.11 ±0.07*
LVESD, mm	2.11 ±0.04	2.69 ±0.09**	2.13 ±0.06	3.29 ±0.14**	1.74 ±0.07	2.68 ±0.11**
% FS	48.42 ±1.14	36.73 ±0.89**	48.12 ±0.84	26.76 ±1.78**	53.00 ±1.68	34.85 ±1.76**
PAV, M/s	0.992 ±0.029	0.922 ±0.033	0.902 ±0.044	0.802 ±0.037	0.875 ±0.048	0.809 ±0.044
Vcfc, circ/s	9.49 ±0.48	7.28 ±0.45*	9.46 ±0.26	4.93 ±0.33**	10.34 ±0.81	6.16 ±0.31**
LVM, mg	95.69 ±1.88	95.50 ±2.28	102.41 ±4.00	100.49 ±3.39	92.27 ±2.02	89.60 ±1.54
LVM/BW, mg/g	3.32 ±0.08	3.53 ±0.18	3.19 ±0.10	3.32 ±0.15	3.29 ±0.37	3.30 ±0.09

*p < 0.05 ace2^{-y} vs ace2^{+/y} or ace2^{-y} vs ace2^{+/y}**p < 0.01 ace2^{-y} vs ace2^{+/y} or ace2^{-y} vs ace2^{+/y}

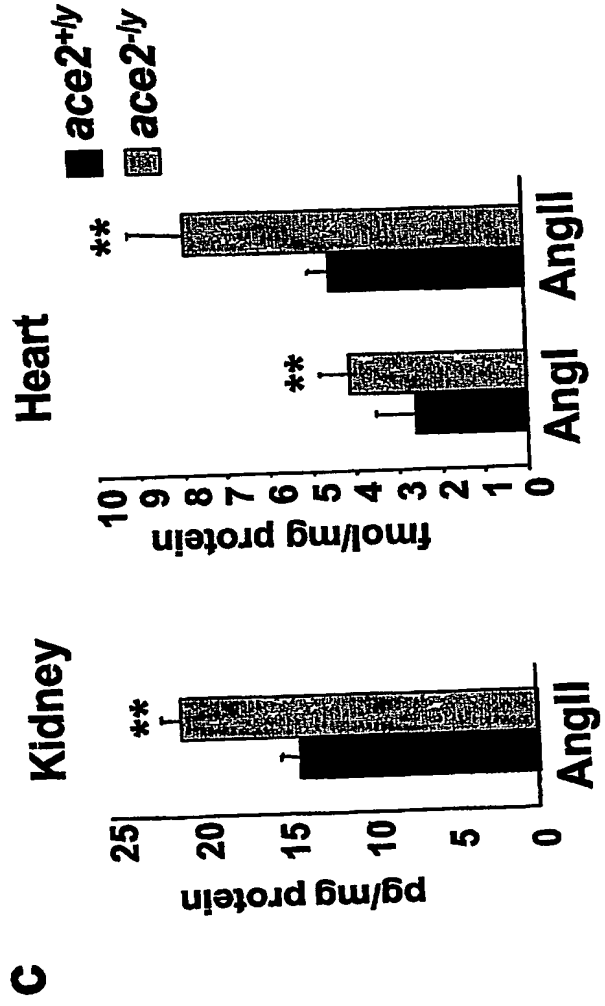
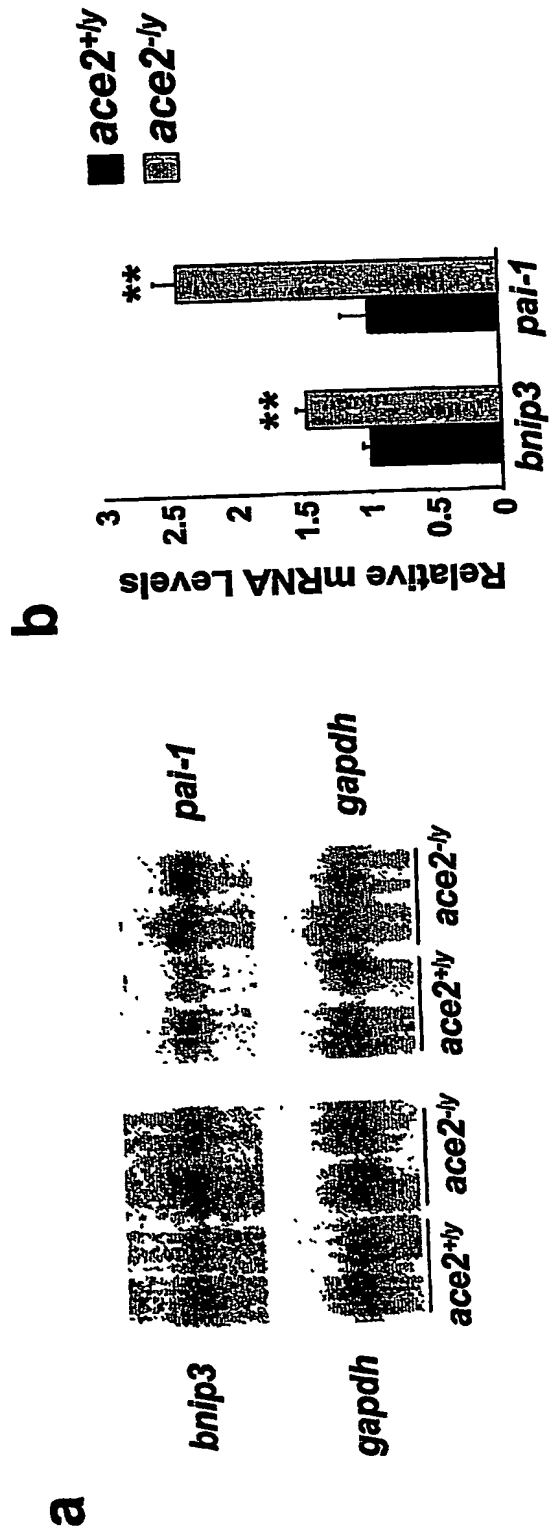
Bpm = heart beats per minute; AW = anterior wall thickness; LVEDD = left ventricle end diastolic dimension; LVESD = left ventricle end systolic dimension; %FS = percent fractional shortening; PAV = peak aortic outflow velocity; Vcfc = Velocity of circumferential fiber shortening; LVM = calculated left ventricular mass; BW = body weight.

Table 2. Invasive hemodynamic parameters of 6 months old ace2^{ly} mice

	ace2 ^{ly}	ace2 ^{ly}
	N = 8	N = 8
Heart rate, bpm	303 ±16	298 ±8
SBP, mmHg	111.5 ±2.4	91.6 ±3.0
DBP, mmHg	70.5 ±3.0	50.3 ±2.4**
MBP, mmHg	84.2 ±2.8	64.0 ±2.6**
LVSBP, mmHg	107.4 ±4.5	87.5 ±2.3**
LVEDBP, mmHg	5.5 ±0.8	5.3 ±0.7
dP/dT max	5579 ±422	3034 ±124**
dP/dT min	-5055 ±257	-2929 ±271**

**p < 0.01 ace2^{ly} vs ace2^{ly}

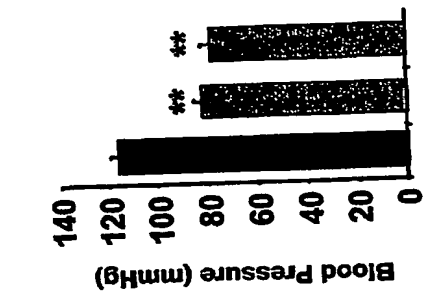
SBP = systolic blood pressure; DBP = diastolic blood pressure; MBP = mean arterial blood pressure; LVSBP = left ventricle systolic blood pressure; LVEDBP = left ventricle end diastolic blood pressure; dP/dT max = maximum 1st derivative of the change in left ventricular pressure/time; dP/dT min = minimum 1st derivative of the change in left ventricular pressure/time



Crackower Figure 7

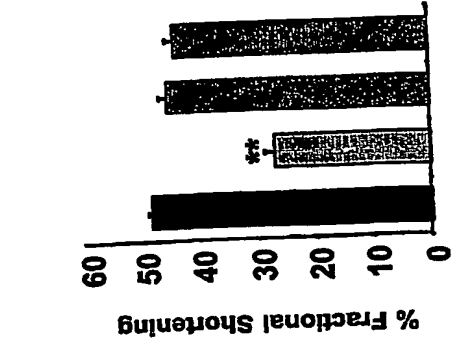
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 ■ **ace^{-/-}**
 ■ **ace^{-/-}**
 ■ **ace2^{-ly}**

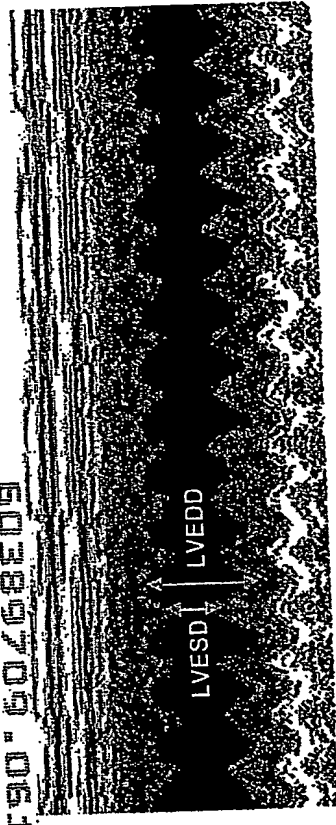
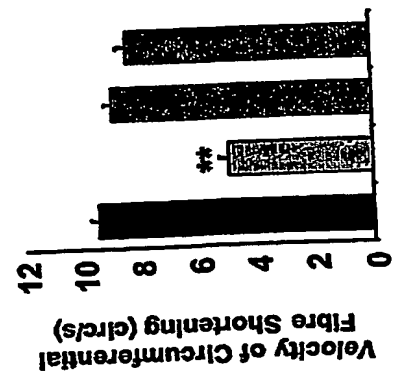


a

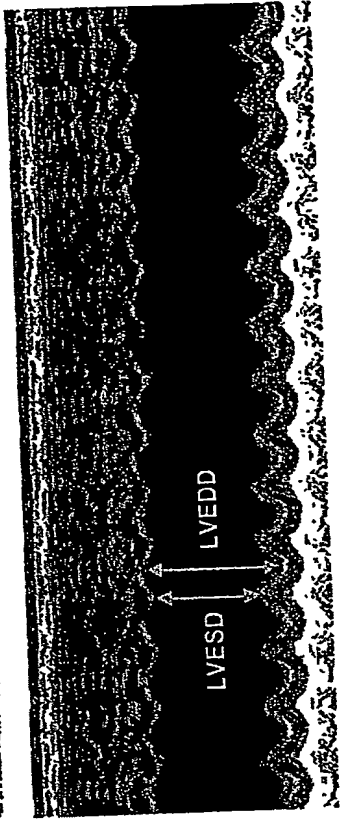
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 ■ **ace2^{-ly}**
 ■ **ace^{-/-}**
 ■ **ace^{-/-}**
 ■ **ace2^{-ly}**



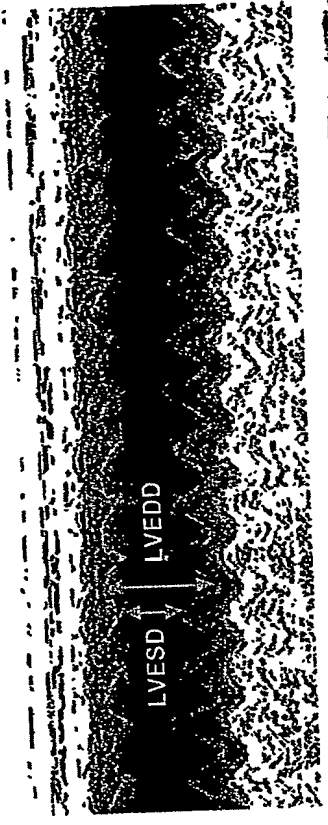
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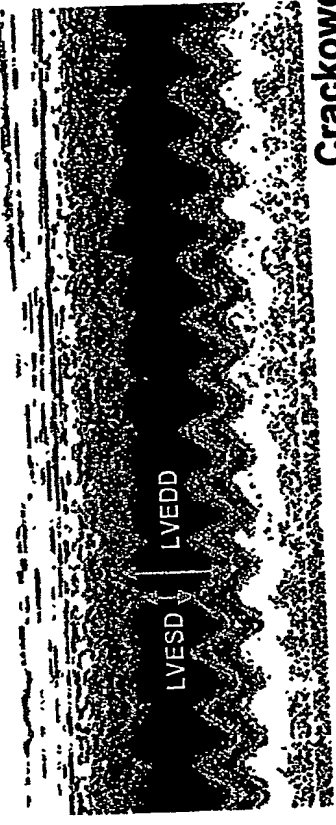
ace2^{+/y}



ace2^{-ly}



ace^{-/-}



ace^{-/-}
ace2^{-ly}

Crackower Figure 8

Table 3. Heart functions of 6 months old ace and ace/ace2 null mice

	ace ^{-/-}	ace ^{-/-} ace2 ^{-/-}
	n=8	n=8
Heart Rate, bpm	507 ±17	491 ±10
AW, mm	0.63 ±0.01	0.65 ±0.02
LVEDD, mm	3.86 ±0.04	3.79 ±0.07
LVESD, mm	2.11 ±0.04	2.13 ±0.07
% FS	45.34 ±1.11	43.95 ±1.24
PAV, M/s	0.985 ±0.064	0.931 ±0.040
V _{dc} , circ/s	8.94 ±0.25	8.40 ±0.27

AW = anterior wall thickness; LVEDD = left ventricle end diastolic dimension;
 LVESD = left ventricle end systolic dimension; %FS = percent fractional
 shortening; PAV = peak aortic outflow velocity; V_{dc} = Velocity of
 circumferential fiber shortening.

Figure 9a

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(Figure 9a continued)

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Figure 10a

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Figure 10b

MSSSSWLLLSLVAVTTAQSLTEENAKTFLNNFNQEAEDL3YQSSLASWNYNTNITEENAOKMSEAAKWSAFYEEQSKTAQSF
SLQETQTPIIKRQLOALQSGSSALSADKNKQLNTILNTMSTIYSTGKVCNPKNPQECLLLEPGLDETMATSTDYNSRLWAVE
GWRAEVKGQLRPLYEEYVVLKNEMARANNYNDYGDYWRGDYAEAGDGYNYNRNQLIEDVERTFAEIKPLYEHLHAYVRRKLM
DTYPSYISPTGCLPAHLLGDMWGRFTNLPLTVPFAQKPNIDVTDAMMNCGWDAERITQEAKEFFVSVGLPHMTQGFWANSM
LTEPADGRKVVCHPTAWDLGHGDFRIKMCVKVTMDNFLTAAHEMHHIQYDMAYARQPFLLRNGANEGFHEAVGEIMSLSAATP
KHLKSIGLLPSDFQEDSETEINFLLKQALTIVGTLPTTYMLEKWRWMVERGEIPKEQWMKKWEMKREIVGVVEPLPHDETYC
DPASLEHVSNDYSFIRYYTRTIYQFQFQEAALCOAAKYNGSLHKCDISNSTEAGQKLLKMLSLGNSEPWTKALENVVGARNMDV
KPLLNYFQPLFDWLKEQNRNSFVGWNTWSPYADQSIKVRISLKSALGANAYFTWNNEMFLFRSSVAYAMRKYFSIIKNQTVF
FLEEDVRVSDLKPRVSFYFFVTSPQNVSDVIPRSEVEDAIRMSRGRINDVTGLNDNSLEFLGIHPTLEPPYQPFVTIWLIIFG
VVMALVVVGIIILLIVTGIGRKKKNETKREENPYDSMDIGKGESNAGFQNSDDAQTSE

SEQ ID NO:4

60389709.061902

	African Am		Asian	Caucasian		Reference
rs879922	C(G)	60	100	70	C	TAACTGAAGTCCAAAAACATATGTTCTTACCTA(C/G)TAACCCAGT CCTGAATTTGCTGGAGCTCAGTTT SEQ ID NO. 5 GTATCTCACCATCAGAAACACAAGCTTGTTGTC/TAAGGATATTAGCTA ATAAAGTTTGTAACaI SEQ ID NO. 8 CCATGAGTTCTAGCCAGACTTTCTTCAACCCAGCACCTGCTCCC(C/G)TT TACCAGAGAGCATTCTCAGACCACAAGATCC SEQ ID NO. 7 Acaggttgctbaaactatcatcagagttagtggaabactgcacatc(C/A) CACTATTGGAATATTCTGGTGTATCTTTGTATTAAATTTCTCAGTGGG T SEQ ID NO. 8 Atgtgccactgcctcagctaggtagcagagcaagadtc(C/T) GTTTCAAAAAAAGGAAATATACACC SEQ ID NO. 9 CTTTGGAAACCTGTTTAAACCAAGCTTTTTTCCATATCTCTATCTGAT GGAG(C/T)TCTCCACACTTCTACATCAGCAGCTTATGACAC SEQ ID NO. 10 CTTTGGAAACCTGTTTAAACCAAGCTTTTTTCCATATCTCTATCTGAT GG(G/A)CCTCTCCACACTTCTACATCAGCAGCTTATGACAC SEQ ID NO. 11 AACACAGCAGTCACAAATGAATAATGCCAACCAATTTATACATTTCCAC ACTT(G/A)CAAGTCAATTTTCCAAATGGAGCTGTTGATGAACCTAATCTA GGTTGCAAGGCATGAA SEQ ID NO. 12 TTCTTGCCAAATATGATAACTTTTGCCCTTAAACACAGCAGTCACAAATG AATAAAT(G/A)CCAAACCAATTTATACATTTCCACACTTACAACTCAATTT CCAATGGAGCTGTTGATG SEQ ID NO. 13 GAAATCTTGCCAAATATGATAACTTTGCCCTTAAACACAGCAGTCACAA AATGAATAAATACCA(G/A)ACCATTATACATTTCCACACTTACAACTCA AATTTCCAAATGGAGCTGTTGATGAA SEQ ID NO. 14 GAAATCTTGCCAAATATGATAACTTTGCCCTTAAACACAGCAGTCACAA AATGAATAAAT(G/A)CCAAACCAATTTATACATTTCCACACTTACAACTCA TTTCCAAATGGAGCTGTTGATGAA SEQ ID NO. 15 ATAGTCACTAAATGTTATGACACAGGACTATGCT(G/A)TATCTTATAT GATGTTCTTTTATGATATCTG SEQ ID NO. 16 GTTTACAAAGTGTATTCTTCAATTTGAA(C/T)GTCAAGTTTTTCTTTTACA CTTATAGATAAGTACATTTT SEQ ID NO. 17 GTGCTACCTCCCAATGCCAATAGCTTTTATTTGGAAATA(C/T) TACTATAGAGACTTGGTCATAGGACCTGATTCATT SEQ ID NO. 18
rs757066	T(C/T)	100	100	70	T	
rs714205	C(G)	70	50	80	C	
rs328442	C(A/C)	50	80	90	A/C	
rs233574	C(C/T)	80	100	80	C	
rs1978124	C(C/T)	90	100	50	C	
rs1978124-2(new)	G(G/A)	30	70	40	C	
rs1514282	A(A/G)	70	100	100	A	
rs1514282-2(new)	A(A/G)	20	50	30	A	
rs1514281	A(A/G)	70	100	100	A	
rs1514281-2(new)	A(A/G)	20	50	50	A	
rs1514278	A(A/G)	Failed	100	70	A	
rs1514280	C(C/T)	80	100	80	C	
rs233575	C(C/T)	100	100	50	C	

Figure 11

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